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Abstract for Dr. Tarr's final tech report.

The effects of perfluorodecanoic acid (PFDA) on humoral, cellular, and innate immunity in Fischer 344 rats was studied at 8 days and 30 days following a single intraperitoneal injection at doses of mg/kg body weight. Effects of in vitro PFDA treatment were also determined. PFDA was found to significantly decrease IgG2 alpha production at 8 days following PFDA treatment and to cause a trend of decreased DTH responsiveness, reflecting inhibitory effects on humoral and cellular mediated immunity. Innate immunity was assessed by natural killer cell function and macrophage function. A significant increase in NK activity was observed at 50 mg/kg PFDA at 30 but not at 8 days following PFDA treatment. PFDA also suppressed the oxidative burst of macrophages (8 days after PFDA treatment at 20 mg/kg) and phagocytosis. Various mechanisms were examined to account for the observed immunosuppression. The evidence suggests that PFDA suppressed IgG2 alpha production (8 days) and DTH responsiveness through combined underlying mechanisms of cytotoxicity, decreased IL-1 and IL-2 production, increased PGE production, and decreased MHC II expression. Inhibition of phagocytosis and respiratory burst may have resulted from membrane alterations, disrupted energy production, and/or disrupted NADPH oxidase activity. Increased NK activity (50 mg/kg, 30 days) appeared to be due to drug-induced anorexia since similar affects were observed in pair-fed animals. An attempt was also made to use fluorescent indicator dyes and antibodies to measure peroxisome formation in livers of PFDA-treated rats. Neither effort proved to be very successful. The dye lacked specificity and the antibodies developed against enoyl CoA hydratase, a peroxisomal enzyme, were too weak to demonstrate reactivity following 2D gel electrophoresis.

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Flow Cytometric Analysis of Hepatocytes From Normal, PFDA and PH/DEN/PB-Treated Rats

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Air Force Office of Scientific Research
Bolling Air Force Base, D.C. 20332-6448

Grant No. AFOSR-90-0371
Final Report

September 1993

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FINAL PROGRESS REPORT

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Title: Flow Cytometric Analysis of Hepatocytes from Normal, PFDA and PH/DEN/PB-Treated Rats

Principal Investigator: Melinda J. Tarr
Department of Veterinary Pathobiology
The Ohio State University
Columbus, Ohio 43210

Funding Period:

I. STATEMENT OF WORK

The following document is the final progress report for AFOSR-90-0371. The report covers the body of work completed over the last funding period and may include data reported in previous progress reports. Previously reported data is included for the convenience of the reader so that the entire multicomponent study can be presented together in a comprehensive formate. The report contains two broad areas of work: 1) effectds of PFDA on parameters of the immune system, and 2) piolet studies hepatotoxic effects of PFDA treatment.

II. RESEARCH REPORT

A. IN VIVO EFFECTS OF PFDA ON HUMORAL IMMUNITY, CELLULAR IMMUNITY, AND LYMPHOID ORGAN WEIGHTS

1. Introduction

In this study, the effects of PFDA treatment on antibody production and delayed type hypersensitivity (DTH) will be presented. PFDA was injected I.P. at a dose of either 20 mg/kg or 50 mg/kg b.w. in Fischer 344 rats 8 days or 30 days prior to sacrifice. Rats were immunized with the T cell-dependent antigen keyhole limpet hemocyanin (KLH). The effects of PFDA on KLH-specific IgG_{2a}, IgM, and IgA production and the DTH response to intradermally injected KLH were determined. The immunotoxicity of PFDA was evaluated initially as changes in lymphoid organ weights following treatment as has been previously reported by Andersen *et al.* (1981).

Antibody production in response to KLH requires antigen processing and presentation in association with major histocompatability (MHC) Class II molecules by macrophages and/or B cells. Cytokine production is required for T cell activation, and T cell-B cell cooperation is also necessary for antibody production in response to a T cell-dependent antigen. The T cell receptor recognizes the antigen in association with

MHC class II molecules. CD4 receptors on T cells bind to nonpolymorphic determinants of the MHC Class II molecules, CD2 receptors on T cells bind to lymphocyte function-associated antigen (LFA)-3 on B cells; and LFA-1 binds to intercellular adhesion molecule (ICAM)-1 on macrophages (Burstein and Abbas, 1991). Changes in one or a combination of these requirements could alter antibody production.

Intradermal injections of antigen promote the initiation of a T-cell response, resulting in a DTH response. The DTH response, which has a slow onset of 24-48 hours (Stanworth, 1985), requires T cell activation and production of chemotactic factors to induce macrophages to enter the site, to activate them (Macrophage activation factor), and to immobilize them (Macrophage inhibition factor) (Yoshida *et al.*, 1989).

2. Methods and Materials

a. Animals

Male Fischer 344 rats, 7-9 weeks of age, were purchased from Harlan Sprague Dawley. Rats were acclimatized for 1 week at the animal resources facility prior to experimentation. Rats weighed approximately 200 grams at the initiation of the experiments.

b. Materials

PFDA was purchased from the Aldrich Chemical Company (Milwaukee, WI). KLH, used for immunization was purchased from ICN Biomedicals (Costa Mesa, CA). Polyclonal rabbit anti-rat IgG_{2a}, IgM, and IgA were obtained from Bioproducts for Science, Inc. (Indianapolis, IN). Immunoglobulin standards and orthophenylenediamine (OPD) were purchased from Zymed (San Francisco, CA). All other reagents were of analytical grade and obtained commercially.

c. Chemical exposure

Fischer 344 rats were injected I.P. with a single dose of PFDA (20 mg/kg or 50 mg/kg) in 50% propylene glycol/water 8 days or 30 days prior to sacrifice. Two rats were injected for each dose of PFDA per experiment. PFDA has been shown to cause anorexia (Andersen *et al.*, 1981). To discern which effects of PFDA resulted from anorexia, two rats were pair-fed to 20 mg/kg PFDA-treated animals and two rats were pair-fed to 50 mg/kg PFDA-treated animals per experiment. The amount of food consumed by PFDA-treated rats was determined daily, and pair-fed rats received the same amount of food. Pair-fed rats were injected with an equal volume of the same vehicle (50% propylene glycol/water) without PFDA. Two control rats which were fed *ad libitum* were also included per experiment.

d. Immunization

KLH (5 mg/ml) was prepared in sterile normal saline. Rats were injected with KLH (0.2 ml) in the caudal tail fold. The initial injection was at 14 days prior to

sacrifice, and the second injection was at 7 days prior to sacrifice. Non-immunized rats were included in the study to provide negative controls for antibody and delayed type hypersensitivity (DTH).

e. Antibody Production

8 days or 30 days after PFDA treatment, rats were anesthetized with ketamine (50 mg/kg) and xylosine (16 mg/kg) and blood samples were obtained by cardiac puncture. Serum was isolated and stored at -20°C until analyzed. Serum samples were analyzed for KLH-specific IgG_{2a} , IgM, and IgA by enzyme-linked immunosorbent assay (ELISA) as described by Exon *et al.*, 1990. Briefly, ELISA plate wells were coated overnight at 4°C with KLH (2 mg/ml in 0.05M Tris buffer, 0.85% NaCl, pH 9.6) or with the appropriate rabbit anti-rat immunoglobulins at a 1:1000 dilution and were then blocked with 5% fetal calf serum in phosphate buffered saline containing 0.05% Tween. Serum samples or immunoglobulin standards at appropriate dilutions were added to the plates and incubated overnight at 4°C . Serum samples from non-immunized rats (which would not contain anti-KLH antibodies) were used as negative controls to determine levels of nonspecific binding. Plates were washed with saline containing 0.05% Tween and the appropriate horseradish-peroxidase conjugated rabbit anti-rat immunoglobulin antibodies were added at a dilution of 1:5000 followed by incubation at room temperature for 1 hour. After extensive washing, color was developed using OPD in 0.05M citrate phosphate buffer (pH 5.0) containing 0.03% H_2O_2 . The reaction was stopped by the addition of $50\ \mu\text{l}$ H_2SO_4 (10N). Plates were read in a Coulter Microplate Reader (Coulter Electronics, Hialeah, FL) at 490/650 nm and absorbance values were converted to $\mu\text{g/ml}$ (IgG_{2a}) or ng/ml (IgM, IgA) utilizing standard curves. Data are presented as a percentage of *ad libitum*-fed control values. Serum samples were collected from two animals per treatment group per experiment. The assay was performed in duplicate and data was analyzed for 5 experiments. Samples were not averaged prior to analysis.

f. DTH Response

The DTH response was determined as described by Exon *et al.*, 1990. Briefly, heat-aggregated KLH was prepared at a concentration of 20 mg/ml in sterile normal saline by heating at 80°C for 1 hour. Heat-aggregated KLH (0.1 ml) was injected 24 hours prior to sacrifice into the right hind footpad of rats which were previously immunized against KLH. Equal volumes of normal saline were injected into the left hind footpad of these rats as an internal control. The same treatment was given to non-immunized rats as a negative control for swelling due to trauma from injection. Footpad swelling was measured with vernier calipers. The DTH response was equal to the difference between swelling in the KLH-injected footpads and swelling in the normal saline-injected footpads. Data from 5 experiments are presented, measurements were made on 2 animals per treatment group. Data were not averaged prior to analysis.

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g. Lymphoid Organ Weights

Spleen and thymus weights were determined and relative organ weights were calculated (organ weight/body weight) to account for decreases in body weight amongst PFDA-treated and pair-fed rats.

h. Statistical Analysis

All data were analyzed by ANOVA using SAS^R software (SAS Institute). Treatment groups that differed significantly from each other ($p < 0.05$), data were then analyzed by Tukey's studentized range test for differences between groups. Treatment groups were considered to be statistically different from controls when $p < 0.05$.

3. Results

a. PFDA-induced Anorexia

Ad libitum-fed rats consumed an average of 17-20 grams of Purina Rat Chow per day. Food consumption was decreased in rats which were treated with 20 mg/kg PFDA by 17-18% from days 1-3 (n.s., Figure 1). Subsequently, food consumption in these rats recovered to near control values. Although there were day to day fluctuations in food consumption, overall food consumption was decreased in rats which were treated with 50 mg/kg PFDA by 30-70% from days 1-21 ($p < 0.05$ except days 11, 17, 25, 27, 29) after which food consumption recovered to near control values. Shown is mean food consumption for 4 representative 8 day and 4 representative 30 day experiments, 2 animals per treatment group. Data were not averaged prior to statistical analysis.

b. Antibody Production

KLH-specific IgG_{2a} levels at 8 days after PFDA treatment were significantly decreased by an average of 54% ($210 \pm 157.3 \mu\text{g/ml}$, $p < 0.05$) at a dose of 20 mg/kg; and by an average of 69% ($95.8 \pm 52.1 \mu\text{g/ml}$, $p < 0.05$) at a dose of 50 mg/kg when compared to *ad libitum*-fed controls ($850.1 \pm 960.9 \mu\text{g/ml}$, Figure 2, top). KLH-specific IgG_{2a} levels at 8 days after PFDA treatment were also significantly less than pair-fed controls (by 59% at 20 mg/kg and by 75% at 50 mg/kg). At 8 days after PFDA treatment with 20 mg/kg or 50 mg/kg, KLH-specific IgM ($227.3 \pm 92.1 \text{ ng/ml}$ at 20 mg/kg; $234.0 \pm 149.7 \text{ ng/ml}$ at 50 mg/kg, Figure 3, top) and IgA levels ($598.5 \pm 422.3 \text{ ng/ml}$ at 20 mg/kg; $485.0 \pm 470.0 \text{ ng/ml}$ at 50 mg/ml, Figure 4, top) were not significantly different (n.s.) from *ad libitum*-fed ($296.4 \pm 157.0 \text{ ng/ml}$ IgM; $910.6 \pm 666.6 \text{ ng/ml}$ IgA) or pair-fed controls ($259.8 \pm 146.2 \text{ ng/ml}$ IgM, 20 mg/kg pair-fed; $255.3 \pm 140.3 \text{ ng/ml}$ IgM, 50 mg/kg pair-fed; $738.8 \pm 410.2 \text{ ng/ml}$ IgA, 20 mg/kg pair-fed; $696.9 \pm 718.1 \text{ ng/ml}$ IgA, 50 mg/kg pair-fed).

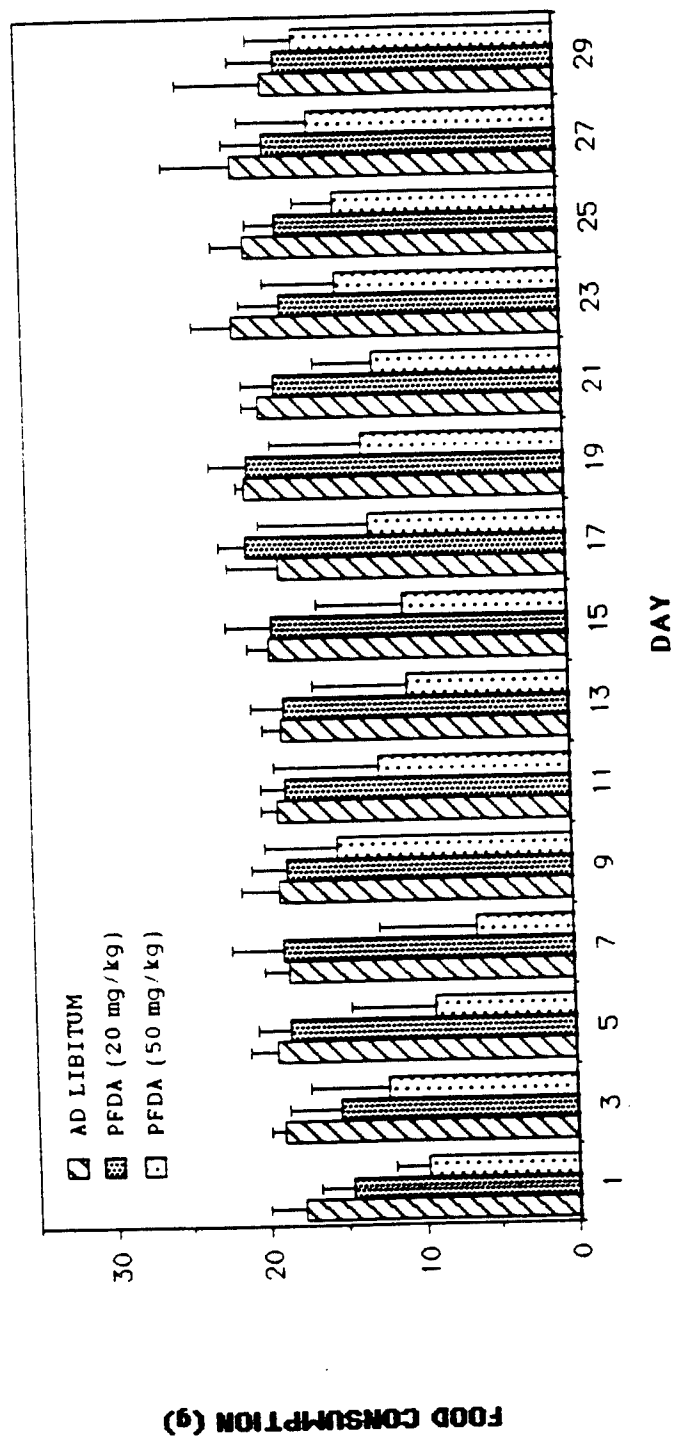


Figure 1: Effect of PFDA on Food Consumption. Rats were injected with PFDA at a dose of 20 mg/kg or 50 mg/kg at 8 days or 30 days prior to euthanasia. Food consumption was monitored daily. Shown is mean food consumption \pm s.d. for four 8 day and four 30 day experiments.

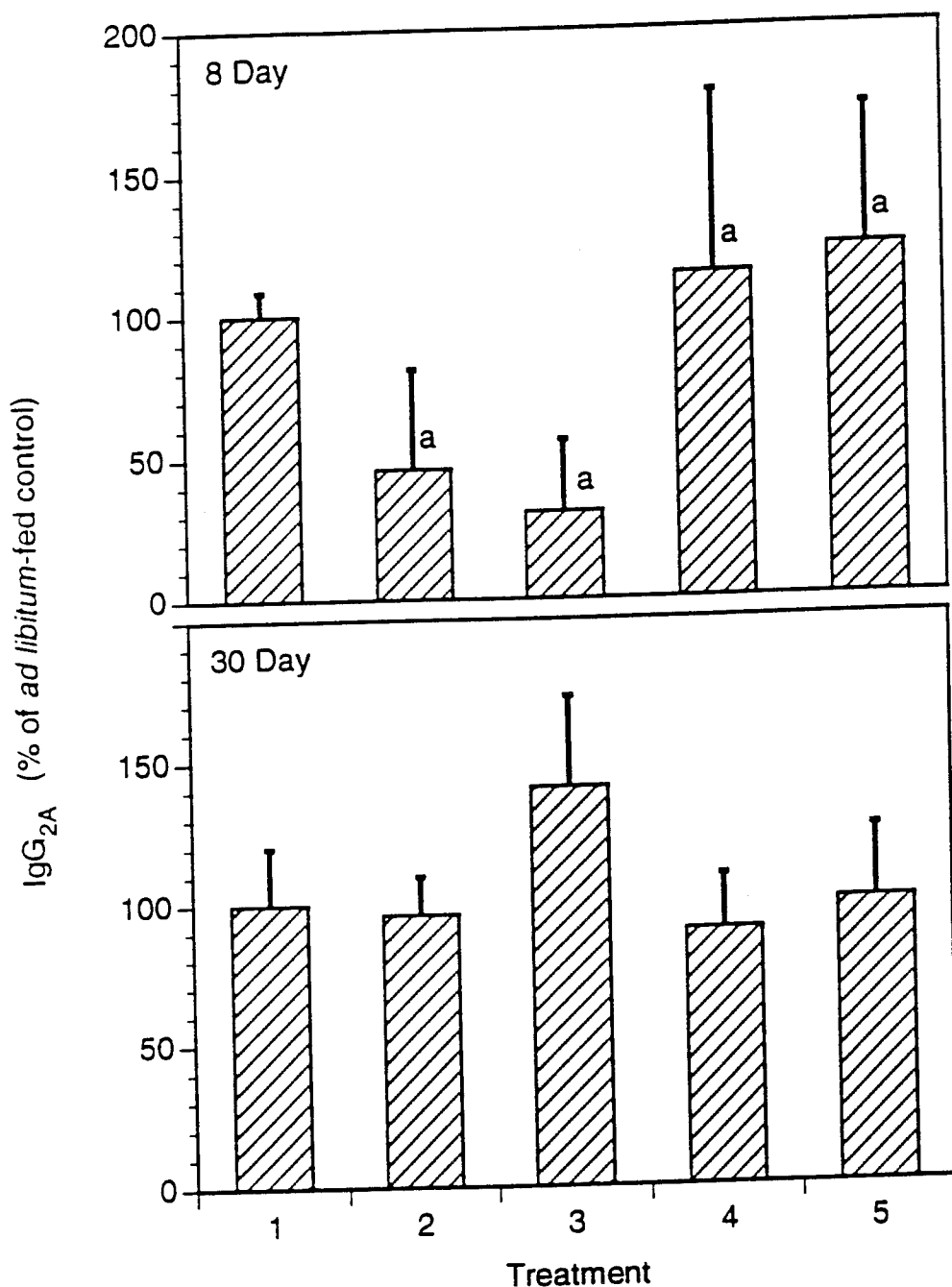


Figure 2: IgG_{2A} Levels After PFDA Treatment. Rats were injected with PFDA at a dose of 20 mg/kg or 50 mg/kg at 8 days (top) or 30 days (bottom) prior to euthanasia. Rats were immunized with KLH and serum anti-KLH IgG_{2A} levels were determined by ELISA. IgG_{2A} levels are expressed as a percentage of the mean *ad libitum*-fed control level. Mean \pm s.d. is shown for 5 experiments. Treatment 1 = *ad libitum*-fed control. Treatment 2 = PFDA (20 mg/kg). Treatment 3 = PFDA (50 mg/kg). Treatment 4 = Pair-fed (20 mg/kg). Treatment 5 = Pair-fed (50 mg/kg). (a) Significantly different from the *ad libitum*-fed control by Tukey's range test (p < 0.05).

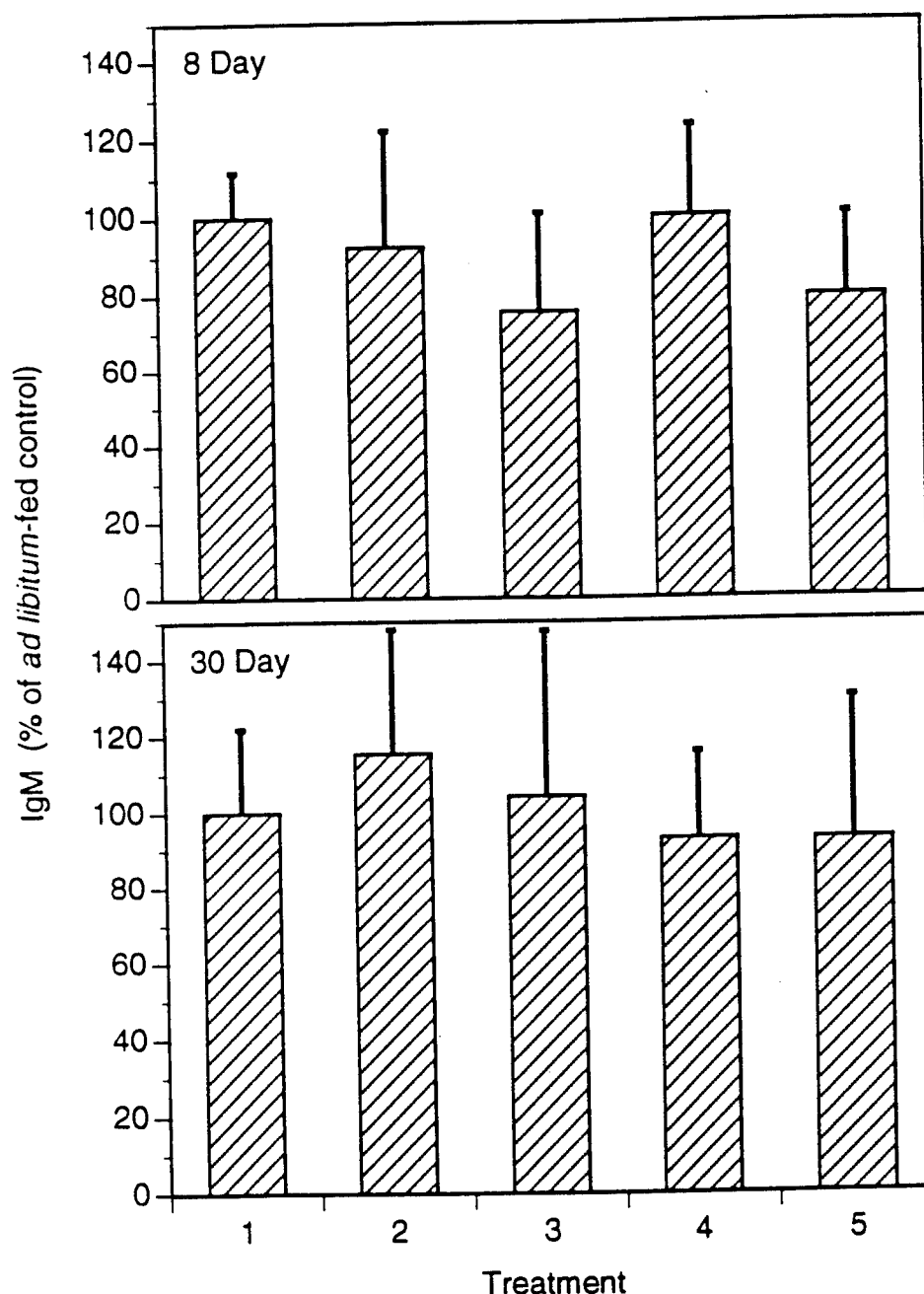


Figure 3: IgM Levels After PFDA Treatment. Rats were injected with PFDA at a dose of 20 mg/kg or 50 mg/kg at 8 days (top) or 30 days (bottom) prior to euthanasia. Rats were immunized with KLH and serum anti-KLH IgM levels were determined by ELISA. IgM levels are expressed as a percentage of the mean *ad libitum*-fed control level. Mean \pm s.d. is shown for 5 experiments. Treatment 1 = *ad libitum*-fed control. Treatment 2 = PFDA (20 mg/kg). Treatment 3 = PFDA (50 mg/kg). Treatment 4 = Pair-fed (20 mg/kg). Treatment 5 = Pair-fed (50 mg/kg).

KLH-specific IgG_{2a} levels at 30 days after PFDA treatment with either 20 mg/kg ($647.9 \pm 243.3 \mu\text{g/ml}$) or 50 mg/kg ($801.2 \pm 324.1 \mu\text{g/ml}$) were not significantly different from *ad libitum*-fed ($748.6 \pm 314.0 \mu\text{g/ml}$) or pair-fed controls ($695.6 \pm 264.2 \mu\text{g/ml}$, 20 mg/kg pair-fed; $768.7 \pm 401.8 \mu\text{g/ml}$, 50 mg/kg pair-fed), although there was a slight increase in the level of IgG_{2a} at the 50 mg/kg dose of PFDA (Fig. 2, bottom). KLH-specific IgM levels 30 days following PFDA treatment at a dose of 20 mg/kg ($468.6 \pm 185.1 \text{ ng/ml}$) or 50 mg/kg ($419.8 \pm 190.6 \text{ ng/ml}$, Figure 3, bottom) were not significantly different from *ad libitum*-fed ($420.4 \pm 180.7 \text{ ng/ml}$) or pair-fed controls ($477.2 \pm 220.0 \text{ ng/ml}$, 20 mg/kg pair-fed; $355.1 \pm 107.1 \text{ ng/ml}$, 50 mg/kg pair-fed). KLH-specific IgA levels (Figure 4, bottom) at 30 days after PFDA treatment at a dose of 20 mg/kg ($116.3 \pm 83.8 \text{ ng/ml}$) or 50 mg/kg ($136.3 \pm 63.4 \text{ ng/ml}$) were not significantly different from *ad libitum*-fed ($218.4 \pm 175.3 \text{ ng/ml}$) or pair-fed controls ($161.0 \pm 116.5 \text{ ng/ml}$, 20 mg/kg pair-fed; $99.3 \pm 51.8 \text{ ng/ml}$, 50 mg/kg pair-fed).

c. DTH Response

The DTH response at 8 days after PFDA treatment was decreased by an average of 40% at a dose of 20 mg/kg (7 out of 9 rats) and by an average of 46% at a dose of 50 mg/kg (5 out of 8 rats) when compared to *ad libitum*-fed controls (Figure 5, top). The DTH response at 8 days after PFDA treatment was also less than pair-fed controls (39%, 20 mg/kg; 41%, 50 mg/kg). These decreases were not statistically significant by Tukey's studentized range test. The DTH response at 30 days after PFDA treatment was decreased by an average of 38% at a dose of 20 mg/kg (5 out of 8 rats), and by an average of 47% at a dose of 50 mg/kg (7 out of 8 rats) when compared to *ad libitum*-fed controls (Figure 5, bottom). The DTH response at 30 days after PFDA treatment was also decreased when compared to pair-fed controls (13%, 20 mg/kg; 40%, 50 mg/kg). These decreases were not statistically significant by Tukey's studentized range test.

d. Lymphoid Organ Weights

1) 8 Day Experiments

i. Thymus At 8 days following PFDA treatment, rats which were treated with 20 mg/kg b.w.. PFDA did not have significantly altered relative thymus weights when compared to *ad libitum*-fed and pair-fed rats (Figure 6). Relative thymus weights in the 50 mg/kg b.w. group were significantly reduced ($p < 0.05$) when compared to *ad libitum*-fed but not pair-fed controls. Pair-fed (50 mg/kg) rats also had significantly reduced relative thymus weight when compared to *ad libitum*-fed controls ($p < 0.05$).

ii. Spleen At 8 days following PFDA treatment, relative spleen weights were significantly reduced in 20 mg/kg and 50 mg/kg b.w. treated rats when compared to both *ad libitum*-fed and pair-fed animals (Figure 7). Pair-fed rats had similar spleen weights when compared to *ad libitum*-fed rats.

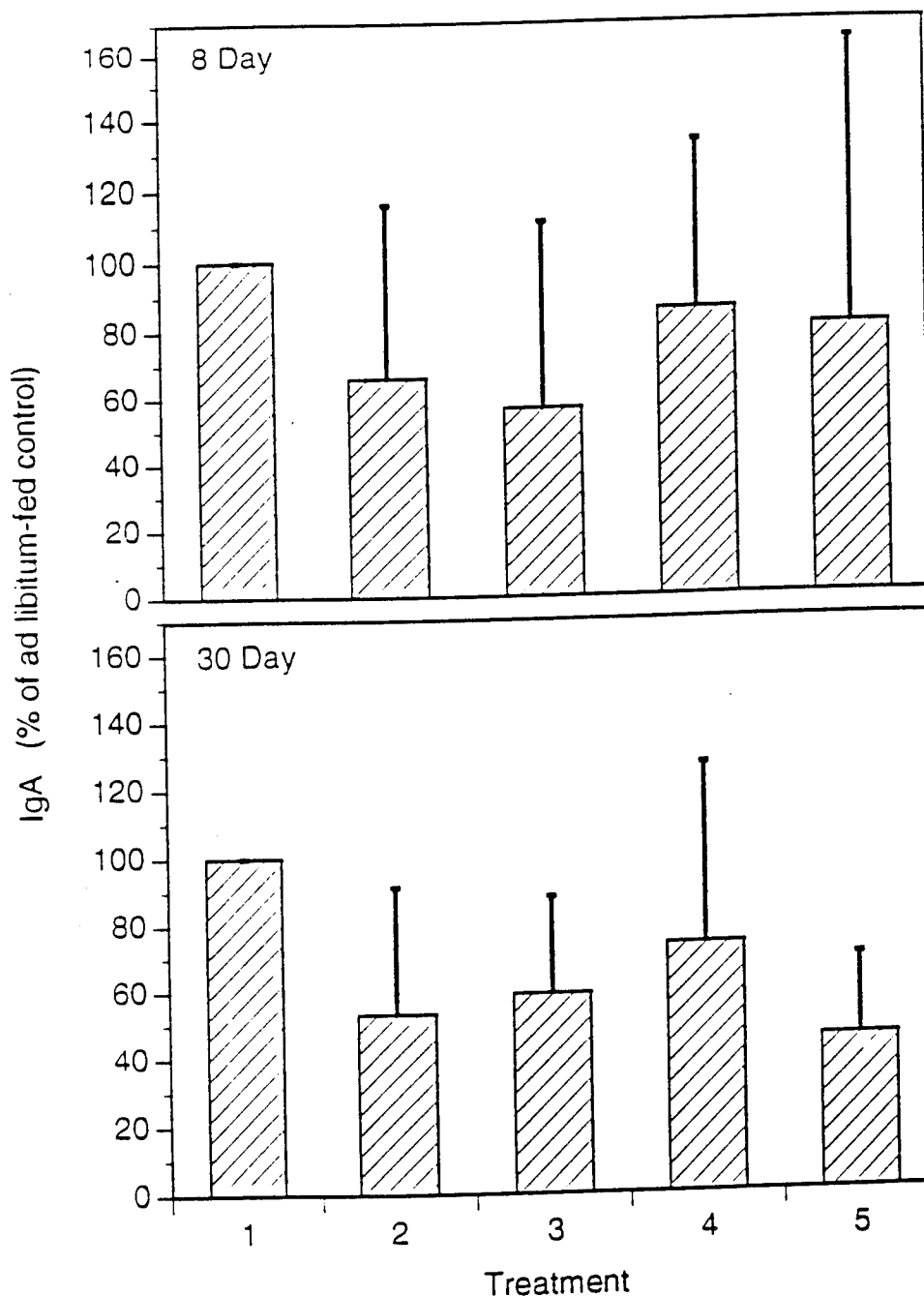


Figure 4: IgA Levels After PFDA Treatment. Rats were injected with PFDA at a dose of 20 mg/kg or 50 mg/kg at 8 days (top) or 30 days (bottom) prior to euthanasia. Rats were immunized with KLH and serum anti-KLH IgA levels were determined by ELISA. IgA levels are expressed as a percentage of the mean *ad libitum*-fed control level. Mean \pm s.d. is shown for 5 experiments. Treatment 1 = *ad libitum*-fed control. Treatment 2 = PFDA (20 mg/kg). Treatment 3 = PFDA (50 mg/kg). Treatment 4 = Pair-fed (20 mg/kg). Treatment 5 = Pair-fed (50 mg/kg).

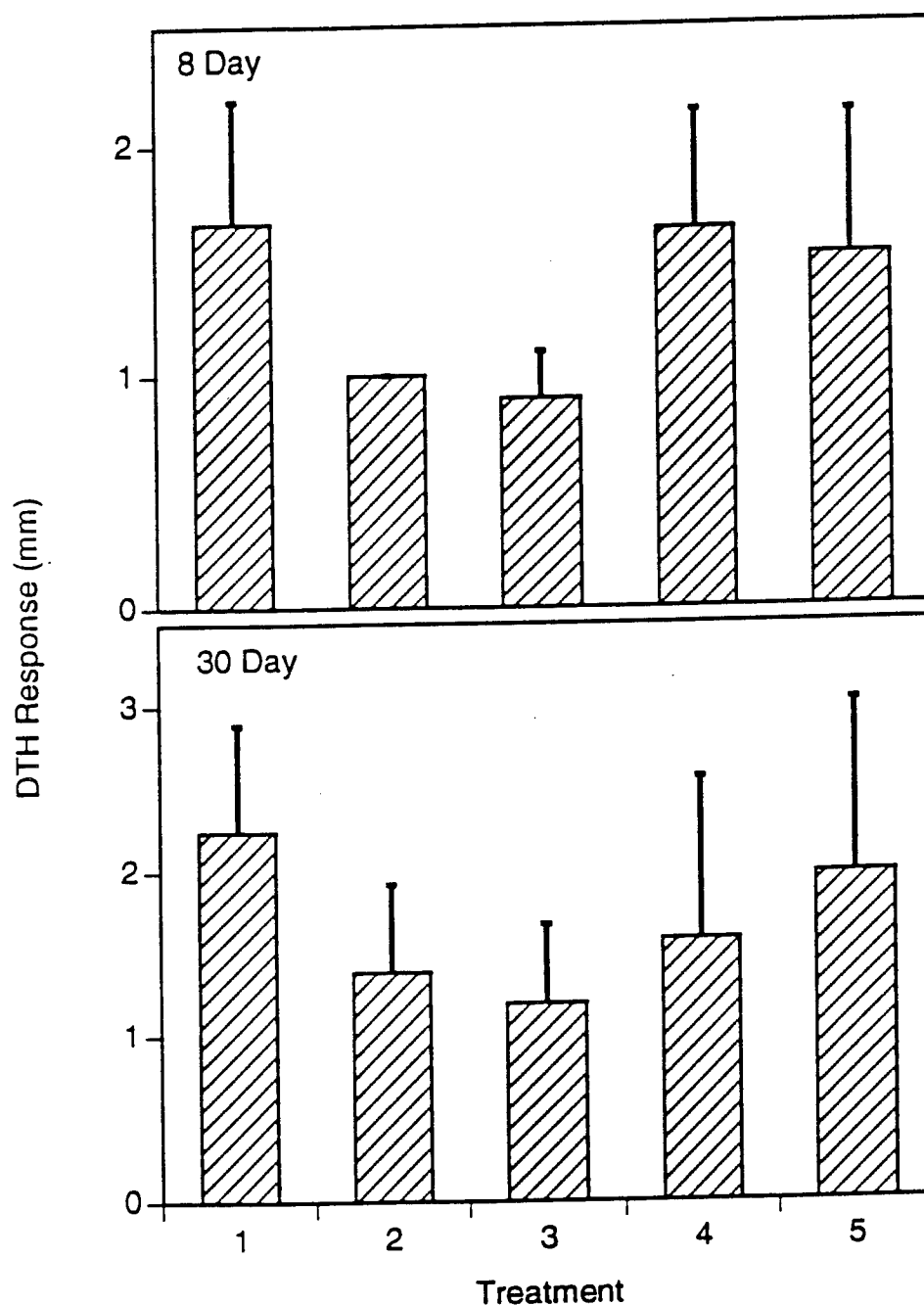


Figure 5: Delayed Type Hypersensitivity (DTH) After PFDA Treatment. Rats were injected with PFDA at a dose of 20 mg/kg or 50 mg/kg at 8 days (top) or 30 days (bottom) prior to euthanasia. Rats were immunized with KLH and subsequently challenged with heat-aggregated KLH in the footpad. Footpad thickness was measured with Vernier calipers. Shown is mean footpad thickness (mm) \pm s.d. for 5 experiments. Treatment 1 = *ad libitum*-fed control. Treatment 2 = PFDA (20 mg/kg). Treatment 3 = PFDA (50 mg/kg). Treatment 4 = Pair-fed (20 mg/kg). Treatment 5 = Pair-fed (50 mg/kg).

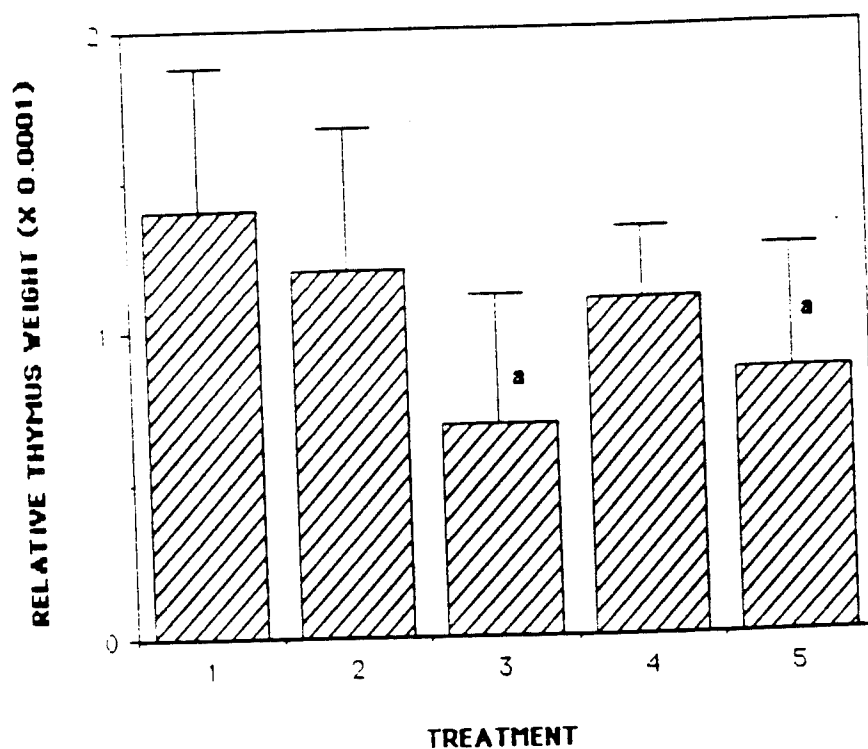


Figure 6: Thymus Weights 8 Days Following PFDA Treatment. Rats were injected with PFDA at a dose of 20 mg/kg or 50 mg/kg at 8 days prior to euthanasia. Organs were removed aseptically and weighed. Shown is mean relative organ weight (Organ weight/body weight) for 8 animals. Treatment 1 = *ad libitum*-fed control. Treatment 2 = PFDA (20 mg/kg). Treatment 3 = PFDA (50 mg/kg). Treatment 4 = Pair-fed (20 mg/kg). Treatment 5 = Pair-fed (50 mg/kg). (a) Significantly different from *ad libitum*-fed control ($p < 0.05$, Tukey's Range Test).

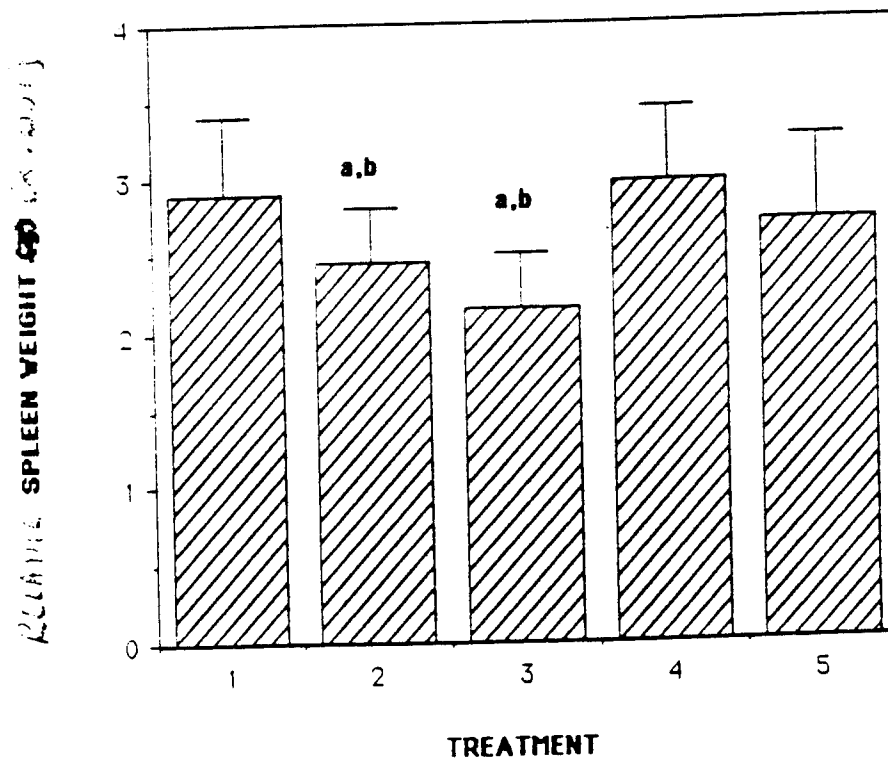


Figure 7: Spleen Weights 8 Days Following PFDA Treatment. Rats were injected with PFDA at a dose of 20 mg/kg or 50 mg/kg at 8 days prior to euthanasia. Organs were removed aseptically and weighed. Shown is mean relative organ weight (Organ weight/body weight) for 8 animals. Treatment 1 = *ad libitum*-fed control. Treatment 2 = PFDA (20 mg/kg). Treatment 3 = PFDA (50 mg/kg). Treatment 4 = Pair-fed (20 mg/kg). Treatment 5 = Pair-fed (50 mg/kg). (a) Significantly different from *ad libitum*-fed control, (b) Significantly different from pair-fed control ($p < 0.05$, Tukey's Range Test).

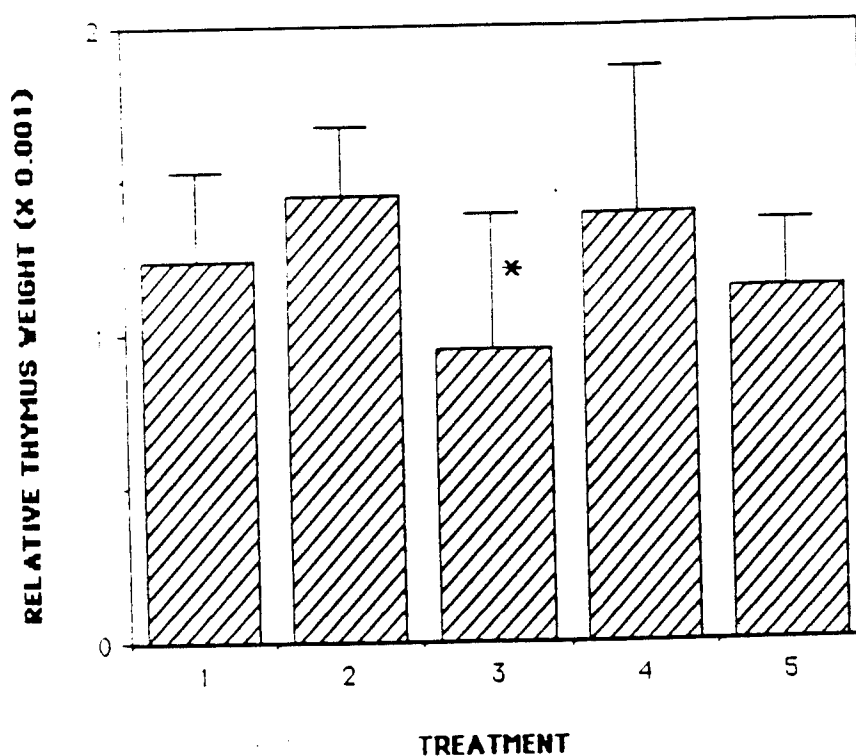


Figure 8: Thymus Weights 30 Days Following PFDA Treatment. Rats were injected with PFDA at a dose of 20 mg/kg or 50 mg/kg at 30 days prior to euthanasia. Organs were removed aseptically and weighed. Shown is mean relative organ weight (Organ weight/body weight) for 6 animals. Treatment 1 = *ad libitum*-fed control. Treatment 2 = PFDA (20 mg/kg). Treatment 3 = PFDA (50 mg/kg). Treatment 4 = Pair-fed (20 mg/kg). Treatment 5 = Pair-fed (50 mg/kg). * = Significantly different from *ad libitum*-fed controls ($p < 0.05$, Tukey's Range Test).

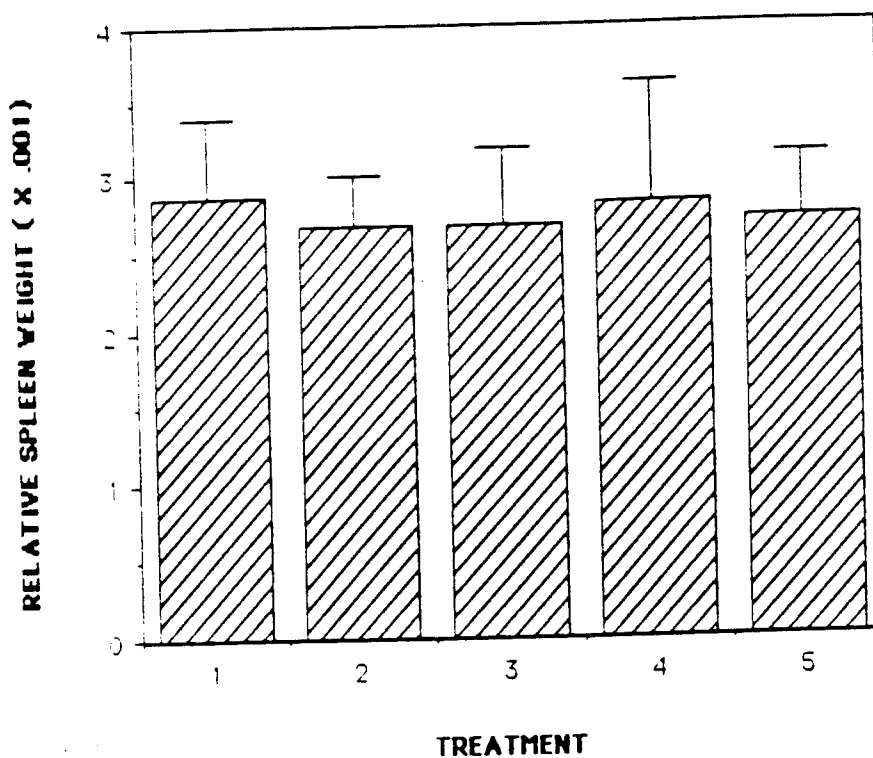


Figure 9: Spleen Weights 30 Days Following PFDA Treatment. Rats were injected with PFDA at a dose of 20 mg/kg or 50 mg/kg at 30 days prior to euthanasia. Organs were removed aseptically and weighed. Shown is mean relative organ weight (Organ weight/body weight) for 6 animals. Treatment 1 = *ad libitum*-fed control. Treatment 2 = PFDA (20 mg/kg). Treatment 3 = PFDA (50 mg/kg). Treatment 4 = Pair-fed (20 mg/kg). Treatment 5 = Pair-fed (50 mg/kg).

2) 30 Day Experiments

i. Thymus

At 30 days following PFDA treatment, relative thymus weight was reduced at 50 mg/kg when compared to both *ad libitum*-fed ($p < 0.05$) and pair-fed animals (n.s., Figure 8).

ii. Spleen

At 30 days following PFDA treatment, spleen weights were similar in 20 mg/kg and 50 mg/kg b.w. treated rats when compared to both *ad libitum*-fed and pair-fed animals (Figure 9).

4. Discussion

The effect of PFDA on humoral immunity and cellular immunity was studied. The effect of PFDA on terminal KLH-specific IgG_{2a} levels was dependent on the time interval between PFDA administration and immunization with KLH. Rats which were treated with PFDA 8 days prior to sacrifice (one day prior to the second immunization) had decreased terminal KLH-specific IgG_{2a} levels when compared to *ad libitum*-fed and pair-fed controls. Thus, the effect of PFDA on IgG_{2a} levels was not due to drug-induced anorexia nor was it due to propylene glycol administration. In contrast, rats which were treated with PFDA 30 days prior to sacrifice (sixteen days prior to the first immunization) did not have decreased terminal KLH-specific IgG_{2a} levels when compared to *ad libitum*-fed and pair-fed controls. This lack of suppression of immunoglobulin production following the longer time interval was probably due to recovery from the initial insult and/or a decrease in the serum concentration of PFDA at the time of immunization. PFDA is eliminated from the body in the feces and it is specifically accumulated in the liver (Ylinen and Auriola, 1990), thereby decreasing the amount of PFDA available to alter IgG_{2a} production.

The mechanism by which PFDA alters IgG_{2a} production is being investigated. PFDA, which is directly toxic for thymocytes *in vivo* (George and Andersen, 1986), may also be directly toxic to B lymphocytes. Alternatively, PFDA may disrupt cytokine production. An elevation of prostaglandin of the E series (Rappaport and Dodge, 1982) or tumor necrosis factor (Kashiwa *et al.*, 1987) may decrease antibody production either directly or through a decrease in interleukin-2 production. Another effect of altered cytokine production could be the inhibition of class switching. The observation that PFDA had no effect on KLH-specific IgM production 8 days after treatment, whereas it decreased IgG_{2a} production supports this hypothesis. Interferon gamma (IFN γ) has been implicated in the induction of IgG_{2a} switching (Coffman *et al.*, 1988) and therefore, reduced IFN γ production could be involved in this process. PFDA did not alter serum IgA levels either, however this can possibly be due to an effect similar to that observed upon TCDD exposure. TCDD, which has similar hepatotoxic effects to those reported in PFDA-treated rats, demonstrates reduced

active transport in the liver of IgA from the serum to the bile (Moran *et al.*, 1986). Normal clearance of IgA would be seen in *ad libitum*-fed and possibly pair-fed controls, and if PFDA reduced this clearance, this would mask PFDA-induced decreases in IgA production by keeping serum levels elevated. Alternatively, defective induction or cytolysis of T and B memory cells could be involved.

Although PFDA did not cause a statistically significant change in DTH response (as determined by Tukey's studentized range test), PFDA did cause a trend of reduced DTH responsiveness at 8 days and 30 days following treatment in the majority of animals when compared to *ad libitum*-fed and pair-fed controls. PFDA apparently causes a longer-term effect on cellular immunity than on humoral immunity since a decrease in the DTH response is still observed 30 days after PFDA treatment. The precise mechanism by which PFDA alters the DTH response is not yet known. It is possible that the circulating T cell population may be decreased by PFDA treatment or lymphokine production and macrophage recruitment may be reduced. PFDA, like other perfluorocarbons (Bucala *et al.*, 1983), may also be directly cytotoxic to macrophages. Though the observed decrease in DTH response was not statistically significant, these data provide preliminary evidence that PFDA may cause a defect in cellular immunity. Decreased DTH responsiveness was also observed in TCDD-treated animals (Clark *et al.*, 1981). The lack of statistical significance may be attributed to the low level of footpad swelling attainable without adjuvant. The DTH response is not the most sensitive method for assessing cellular immune responses, however, this method was selected because it could be used in an economical multiple immunotoxicity approach similar to that described by Exon *et al.* (1990) reducing required numbers of animals. Future experiments to confirm the effect of PFDA on cellular immunity could include mixed lymphocyte reactions or antigen-specific cytolysis of tumor cells by cytotoxic T cells.

Part of the observed decrease in antibody production and DTH response may be attributed to depletion of lymphocytes from the spleen (at 50 mg/kg PFDA, 8 days following PFDA treatment) and from the thymus (at 50 mg/kg PFDA, 8 days and 30 days following treatment). Other compounds which also cause severe depletion in the thymus such as cortisone, nitrogen mustard, and TCDD have been demonstrated to reduce DTH responses (Cohen and Mokychic, 1954; Germuth, 1956; Clark *et al.*, 1981). In addition, studies on adult thymectomized mice have indicated that the thymus serves as a source of precursor cells which seed other lymphatic organs throughout the life of the individual, demonstrating loss of immune responsiveness with loss of the thymus (Miller, 1962).

B. EFFECTS OF IN VIVO PFDA TREATMENT ON INNATE IMMUNITY

1. Introduction

In this study, PFDA was injected I.P. at a dose of either 20 mg/kg or 50 mg/kg b.w. 8 days or 30 days prior to sacrifice. The effect of PFDA on natural killer (NK) cell cytotoxicity and macrophage function (phagocytosis, respiratory burst, and cytokine production) was determined.

NK cells are responsible for non-MHC (major histocompatibility complex) restricted killing of target cells including tumor cells and virally-infected cells (Storkus and Dawson, 1991). Their importance has been demonstrated in beige mice which lack NK cells and which have increased susceptibility to infection and increased spontaneous tumor generation (Haliotis *et al.*, 1985). Several different chemical carcinogens, including ethyl carbamate (Luebke *et al.*, 1986), Aroclor 1254 (Exon *et al.*, 1985), ethylnitrosourea (Talcott *et al.*, 1984), and 3-methylcholanthrene (Talcott *et al.*, 1990), have been shown to decrease NK cell activity, thus allowing tumor cells to escape surveillance. PFDA is a peroxisomal proliferator, and this class of compounds frequently cause hepatocarcinomas (Rao and Reddy, 1987). PFDA has not been reported in the literature to cause hepatocarcinomas, although PFDA has been shown to cause oxidative DNA damage in rat liver (Takagi *et al.*, 1991). It is of interest to determine whether or not NK activity is altered following treatment with PFDA since it could alter susceptibility to hepatocarcinoma formation as well as viral infection.

Mononuclear phagocytes are an important target for the action of environmental contaminants upon the host since these cells are important in the uptake and metabolism of xenobiotics and since functional alterations in the mononuclear phagocyte system can have profound effects upon the host (Lewis and Adams, 1985). Macrophages are responsible for phagocytosis of bacteria and viral particles and are also capable of non-MHC restricted cytolysis of tumor cells. In addition, macrophages provide a regulatory system that controls the physiology of the inflammatory system, the cellular and humoral arms of the immune system, the coagulation system, and proliferation of numerous cell types in diverse tissues (Lewis and Adams, 1985).

The respiratory burst is an important function for killing bacteria and intracellular parasites (Nathan *et al.*, 1979). The respiratory or oxidative burst of macrophages is characterized by an increase in oxygen uptake, enhanced utilization of glucose via the hexose monophosphate shunt and production of superoxide anions and hydrogen peroxide (Pick and Keisari, 1981). The first reaction in the respiratory burst is a one electron reduction of oxygen to superoxide anion catalyzed by NADPH oxidase. Superoxide anion can then be dismutated to hydrogen peroxide either simultaneously or enzymatically with the enzyme superoxide dismutase (Fridovich, 1983). The most important stimulatory influence in the respiratory burst is IFN γ (Pick, 1985). Tumor necrosis factor alpha (TNF α), Interleukin (IL)-3, and granulocyte-macrophage colony stimulating factor may also stimulate the oxidative burst (Murray, 1988; Weiser,

1987; Ho, 1991; Titus, 1989), whereas IL-4 has been demonstrated to depress the oxidative burst (Ho *et al.*, 1992). PFDA-induced interference with macrophage function could cause increased susceptibility to bacterial and parasitic infections as well as reduced tumor surveillance.

2. Methods and Materials

a. Natural Killer Cell Isolation

Spleens from PFDA-treated, *ad libitum*-fed, and pair-fed rats were isolated and single cell suspensions were prepared. Spleens from 2 identically treated, genetically identical rats were combined for each experiment in order to obtain enough cells for analysis. Four 8 day and four 30 day NK experiments were done. Spleens were mechanically disrupted and cells were passed through #50 mesh screens. Cell clumps were further disrupted by passing them back and forth through 18 gauge needles. Samples were subjected to Ficoll (Pharmacia) density gradient centrifugation at 400 x g for 30 minutes to remove erythrocytes and were washed to remove any remaining Ficoll. Cells were incubated in cRPMI (RPMI 1640 supplemented with 10% fetal calf serum, 10 mM HEPES, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 0.25 μ g/ml amphotericin B) at 37°C in petri dishes for 2 hours to remove contaminating macrophages.

b. Natural Killer Cell Assay

The NK cytotoxicity assay was performed as described by Slezak and Horan (13). Briefly, YAC-1 cells (target cells, ATCC TIB160, Rockville, MD) were stained with the green fluorescent, viable membrane dye PKH-2 according to manufacturer's instructions (Zynaxis, Malvern, PA). Cultures containing 100:1, 50:1, and 25:1 nonadherent spleen effector cells:target cells in a total volume of 200 μ l cRPMI were prepared in triplicate in 96-well round bottomed microtiter plates. Cultures were centrifuged at 50 x g for 5 minutes to promote conjugation and were incubated at 37°C for 4 hours. After the incubation period, cultures were centrifuged at 400 x g for 5 minutes, and 100 μ l of the supernatant was removed. Propidium Iodide (PI, 25 μ g/ml in PBS, Sigma Chemical Co., St. Louis, MO) was added in an equivalent volume (100 μ l) to determine viability. Cells were analyzed on an EPICS 753 flow cytometer (Coulter Electronics, Hialeah, FL) using an exciting wavelength of 488 nm with a laser power of 300 mW. Filters used to select for different parameters were: forward angle light scatter (FALS), PI red fluorescence (635 nm band pass, 550 nm long pass dichroic, and 600 nm short pass dichroic filters), and PKH-2 green fluorescence (525 band pass and 550 long pass dichroic filters). For each sample, log green fluorescence versus log red fluorescence histograms were generated. Non-specific cytotoxicity was defined as the percentage of dead target cells occurring when incubated without effector cells, and was subtracted from specific cytotoxicity which was defined as the percentage of dead target cells following incubation with effector cells over the 4 hour culture period. Each experiment was repeated four times. The data were not averaged prior to analysis.

c. Isolation of Peritoneal Macrophages

Rats were sacrificed by CO₂ asphyxiation and 10 ml Hank's Balanced Salt Solution (HBSS) was injected into the peritoneal cavity. A large drainage hole was prepared and peritoneal cells were washed out with a further 10 ml volume HBSS onto petri dishes. Cells were counted and cells from combined spleens (identical treatment, genetically identical) were suspended in complete RPMI at 1×10^6 cells/ml. Macrophages were adherence purified in 24-well tissue culture plates at 37°C for 2 hours.

d. Measurement of Oxidative Burst and Phagocytosis

Oxidative burst and phagocytosis were estimated by the method described by Hasui *et al.* (1989) with some modification. A 20 mM stock solution of 2',7'-dichlorofluorescein diacetate (DCFH-DA, Molecular Probes, Eugene, OR) was prepared in DMSO. Adherence purified macrophages were incubated overnight at 37°C. Cells were removed from tissue culture plates by the addition of cold complete RPMI (1 ml) and scraping and were transferred (800 μ l) to 12 x 75 mm polypropylene tubes. DCFH-DA (diluted 1:66.7, 200 μ l) was added to each tube to be analyzed for oxidative burst. Cells were incubated with or without phorbol-13-myristate acetate (PMA, 100 μ g/ml, GIBCO, Grand Island, NY) or with PI-stained *staphylococcus aureus* (5×10^7 , PANSORBIN, Calbiochem, La Jolla, CA) for 30 minutes. DCFH-DA is lipid soluble and diffuses into cells where it is deacetylated and trapped in the cytoplasm. The non-fluorescent product dichlorofluorescein (DCFH) is oxidized in the presence of hydrogen peroxide to a green fluorescent compound dichlorofluorescein. The percentage of red and green fluorescent cells and the fluorescence intensity was determined by flow cytometry on an EPICS 753 flow cytometer using an excitation wavelength of 488 nm with a laser power of 200 mW. Filters used to select for different parameters were: FALS, DCF-DA green fluorescence (550 nm long pass dichroic and 525 band pass filters) and PI red fluorescence (600 nm short pass dichroic and 635 band pass filters). 10,000 cells were counted for each treatment group per experiment. The experiments were repeated 4 times.

e. Peritoneal Macrophage Cultures

Adherence purified macrophages were incubated with or without lipopolysaccharide (LPS) (10 μ g/ml, Sigma, St. Louis, MO) for a period of 24 hours at 37°C. Supernatants were harvested and frozen at -70°C until analyzed for levels of prostaglandin E (PGE) and interleukin-1 (IL-1).

f. PGE Production

Supernatants from adherence purified macrophages treated with LPS were analyzed for PGE production in duplicate using a commercially available radioimmunoassay (Incstar, Stillwater, MN) according to manufacturer's instructions. Each experiment was repeated four times. Data were not averaged prior to analysis. Briefly, PGE was converted to PGB by alkalination. Non-radioactive prostaglandins within the supernatants competed with a constant amount of [³H] PGB₁ tracer for

binding sites on PGB₁ antibody which was held at a limiting concentration. Concentrations of PGE were then extrapolated from a standard curve. Controls for nonspecific binding and total counts were included.

g. IL-1 Production

IL-1 levels were estimated by the method described by Larrick *et al.* (1985) with some modification. Briefly, LBRM-33-1A5 (ATCC CRL 8079, Rockville, MD) mouse lymphoma cells (5×10^4 /well) are incubated in LBT medium (10% fetal calf serum, 50 mM 2-mercaptoethanol, 10 mM Hepes, 100 U/ml penicillin, 100 μ g/ml Streptomycin, 0.25 μ g/ml amphotericin B) in 96-well round-bottom microtiter tissue culture plates with a submitogenic concentration of PHA (1:500) and the IL-1 containing supernatants or standard in duplicate for 24 hours at 37°C. LBRM-33-1A5 cells require IL-1 to produce IL-2. Supernatants were filtered (0.2 μ m, Nalgene) and frozen at -70°C until analysis for IL-2 levels using [³H]-thymidine incorporation in the IL-2 dependent CTLL-2 line (Ch. III Methods and Materials).

3. Results

a. Natural Killer (NK) Cell Activity

At 8 days following treatment, PFDA did not alter mean NK cell activity significantly when compared to *ad libitum*-fed controls at either dose (Table 1). Mean NK cell activity of 50 mg/kg pair-fed rats was significantly greater than *ad libitum*-fed control values by 22% and 20% at the 50:1 and 25:1 Effector:Target (E:T) ratios respectively ($p < 0.05$). Mean NK cell activity of 50 mg/kg PFDA-treated rats was significantly decreased by 16% and 18% when compared to pair-fed controls at the 50:1 and 25:1 E:T ratios ($p < 0.05$).

TABLE 1. Natural Killer Cell Activity 8 Days After PFDA Treatment

<u>TREATMENT</u>	<u>100:1 E:T RATIO</u>	<u>50:1 E:T RATIO</u>	<u>25:1 E:T RATIO</u>
AD LIB-FED	28.3 \pm 9.2	19.9 \pm 7.4	13.9 \pm 3.8
PFDA 20 mg/kg	29.8 \pm 10.1	19.9 \pm 6.2	13.9 \pm 4.1
PFDA 50 mg/kg	27.9 \pm 12.9	21.3 \pm 8.3 (b)	14.1 \pm 6.2 (b)
Pair-fed 20 mg/kg	29.1 \pm 10.0	20.3 \pm 5.0	13.0 \pm 5.6
Pair-fed 50 mg/kg	33.1 \pm 12.8	25.4 \pm 11.4 (a)	17.3 \pm 8.7 (a)

Rats were injected I.P. with PFDA (20 mg/kg or 50 mg/kg) at 8 days prior to sacrifice. Nonadherent spleen cells were cultured with YAC-1 target cells at various effector:target (E:T) ratios for 4 hours at 37°C. Percent specific cytotoxicity was determined by flow cytometry following propidium iodide incorporation. Experiments were performed in triplicate, and mean \pm s.d. for 4 experiments is shown. (a) significantly different from *ad lib*-fed control, (b) significantly different from pair-fed control by Tukey's studentized range test ($p < 0.05$).

Mean NK cell activity at a dose of 50 mg/kg 30 days following treatment was significantly greater than the *ad libitum*-fed control values by 19% at the 100:1 E:T ratio and by 16% at the 25:1 E:T ratio ($p < 0.05$, Table 2). Mean pair-fed NK cell activity (20 mg/kg and 50 mg/kg) was also significantly greater than *ad libitum*-fed control values by 17-22% at all E:T ratios. Mean NK cell activity at 20 mg/kg PFDA was significantly lower than pair-fed levels at 100:1 and 25:1 ratios by 13% and 20% respectively ($p < 0.05$). Mean NK cell activity at 50 mg/kg PFDA did not differ significantly from that of the pair-fed control.

TABLE 2. Natural Killer Cell Activity 30 Days After PFDA Treatment

<u>TREATMENT</u>	<u>100:1 E:T RATIO</u>	<u>50:1 E:T RATIO</u>	<u>25:1 E:T RATIO</u>
AD LIB-FED	27.8 \pm 7.1	18.4 \pm 6.9	12.3 \pm 4.2
PFDA 20 mg/kg	29.8 \pm 13.0 (b)	21.0 \pm 12.5	12.6 \pm 8.1 (b)
PFDA 50 mg/kg	34.3 \pm 8.7 (a)	20.8 \pm 6.2	14.8 \pm 5.6 (a)
Pair-fed 20 mg/kg	34.3 \pm 13.8 (a)	22.7 \pm 11.5 (a)	15.8 \pm 7.4 (a)
Pair-fed 50 mg/kg	33.1 \pm 17.1 (a)	21.6 \pm 12.1 (a)	15.7 \pm 8.5 (a)

Rats were injected with PFDA (20 mg/kg or 50 mg/kg) at 30 days prior to sacrifice. Nonadherent spleen cells were cultured with YAC-1 target cells at various E:T ratios for 4 hours at 37°C. Percent specific cytotoxicity was determined by flow cytometry following propidium iodide incorporation. Experiments were performed in triplicate and mean \pm s.d. for 4 experiments is shown. (a) significantly different from *ad lib*-fed control (b) significantly different from pair-fed control by Tukey's studentized range test ($p < 0.05$).

b. Measurement of Oxidative Burst and Phagocytosis

1) 8 Day Experiments

An average of 36% of resting resident peritoneal macrophages from *ad libitum*-fed controls produced hydrogen peroxide at a mean fluorescence intensity of 24 (Table 3). At 8 days following PFDA treatment, the percentage of hydrogen peroxide producing cells and the amount of hydrogen peroxide produced per cell was not significantly decreased. Decreases of 7%-8%, n.s. respectively were observed at 20 mg/kg, and decreases of 17% and 11% (n.s.), respectively were observed at 50 mg/kg when compared to *ad libitum*-fed controls. The percentage of positive cells and the amount of hydrogen peroxide produced per cell was increased, however, by 38% and 8% (n.s.) respectively in 20 pair-fed cells; and by 44% and 17% (n.s.) respectively in 50 pair-fed cells.

Following stimulation with PMA, an average of 65% of macrophages from *ad libitum*-fed controls produced hydrogen peroxide at a mean fluorescence intensity of 27. The percentage of hydrogen peroxide producing cells and the amount of hydrogen peroxide produced per cell was decreased by 3% and 6% (n.s.) respectively at 20 mg/kg, and a significant decrease of 34% and 17% ($p < 0.05$) respectively at 50 mg/kg. A significant decrease (47%) in hydrogen peroxide production was also

Table 3. Oxidative Burst and Phagocytosis 8 Days Following PFDA Treatment

TRT	DCF %GRN P<0.8230	DCF MFI P<0.6604	DCF + PMA %GRN P<0.0011	DCF + PMA MFI P<0.7100	PI.SA. % RED P<0.7867	PI.SA. MFI P<0.7260
<i>Ad lib</i> -fed control	36.1 ± 34.0	23.6 ± 7.7	65.2 ± 16.0	27.4 ± 9.8	88.5 ± 11.1	30.6 ± 3.7
PFDA 20 mg/kg	33.6 ± 29.2	23.8 ± 5.8	63.4 ± 10.0	25.8 ± 6.8	79.4 ± 22.9	32.0 ± 4.2
PFDA 50 mg/kg	29.9 ± 28.7	21.0 ± 2.4	43.1 ± 18.8 ^{a,b}	22.8 ± 3.2	76.8 ± 19.7	28.8 ± 1.8
Pair-fed 20 mg/kg	49.9 ± 44.6	25.6 ± 7.8	82.1 ± 11.4	28.2 ± 7.4	86.6 ± 15.8	30.4 ± 5.0
Pair-fed 50 mg/kg	52.0 ± 42.8	26.6 ± 5.5	81.7 ± 8.7	28.2 ± 5.8	86.1 ± 14.8	30.4 ± 1.7

Fischer 344 rats were treated with 20 mg/kg or 50 mg/kg PFDA 8 days prior to sacrifice. *Ad libitum*-fed and pair-fed controls were included in the study. Peritoneal macrophages were isolated and adherence purified. Cells were incubated with dichlorofluorescein diacetate (DCFHDA) ± phorbol myristate acetate (PMA) ± propidium iodide (PI) labelled *staphylococcus aureus* (SA) for 30 minutes at 37°C. Shown is treatment (TRT) versus mean percent positive (green (GRN) - DCF; red -PI.SA.) and mean fluorescence intensity (MFI). These data represent 4 experiments where cells were combined from 2 genetically identical animals from each group. Fluorescence was measured for 10,000 cells per sample. a = significantly different from *ad libitum*-fed control, b = significantly different from pair-fed control, p < 0.05 by Tukey's range test.

observed at 50 mg/kg ($p < 0.05$) when compared to the pair-fed controls. The percentage of hydrogen peroxide producing cells and the amount of hydrogen peroxide produced per cell was increased, however, by 26% and 3% (n.s.), respectively, for 20 pair-fed animals and by 25% and 3% (n.s.), respectively, for 50 pair-fed animals.

An average of 88% of macrophages from *ad libitum*-fed rats ingested PI-labelled bacteria at a mean fluorescence intensity of 31. The percentage of cells ingesting bacteria was decreased at 8 days following PFDA treatment by 10% at 20 mg/kg (n.s.) and 13% at 50 mg/kg (n.s.). When phagocytosis of PFDA-treated rats was compared to that of pair-fed rats, similar decreases were observed (n.s.). The percentage of cells isolated from pair-fed animals which ingested bacteria was similar to that of *ad libitum*-fed controls.

2) 30 Day Experiments

An average of 47% of resting macrophages from *ad libitum*-fed rats produced hydrogen peroxide at a mean fluorescence intensity of 22 (Table 4). At 30 days following PFDA treatment, the percentage of cells producing hydrogen peroxide and the amount of hydrogen peroxide produced per cell was decreased by 47% (n.s.) and 5% (n.s.) respectively at a dose of 20 mg/kg PFDA. The percentage of cells producing hydrogen peroxide was decreased by 53% at a dose of 50 mg/kg PFDA whereas the amount of hydrogen peroxide produced per cell was increased by 9% above *ad libitum*-fed control values. Similar decreases in the percentages of cells producing hydrogen peroxide were observed between PFDA-treated cells and pair-fed cells (n.s.). The percentage of cells producing hydrogen peroxide and the amount of hydrogen peroxide produced per cell was decreased by 17% (n.s.) and 2% (n.s.) respectively for cells from 20 pair-fed animals and by 17% (n.s.) and 5% (n.s.) respectively in cells from 50 pair-fed animals when compared to *ad libitum*-fed controls.

At 30 days following PFDA treatment, an average of 58% of macrophages from *ad libitum*-fed rats produced hydrogen peroxide following stimulation with PMA at a mean fluorescence intensity of 25. The percentage of cells producing hydrogen peroxide was decreased by 46% (n.s.) at a dose of 20 mg/kg PFDA whereas the amount of hydrogen peroxide produced per cell was increased by 3% (n.s.). The percentage of cells producing hydrogen peroxide was decreased by 50% (n.s.) at a dose of 50 mg/kg whereas the amount of hydrogen peroxide produced per cell was increased by 10% (n.s.). Similar decreases in the percentages of cells producing hydrogen peroxide were observed between cells from PFDA-treated and pair-fed animals (n.s.). There was no change in the percentage of cells producing hydrogen peroxide for cells from 20 pair-fed animals and only a slight decrease of 6% (n.s.) was observed in cells from 50 pair-fed animals. The amount of hydrogen peroxide produced per cell was increased by 4% (n.s.) for cells from 20 pair-fed animals and by 24% (n.s.) for cells from 50 pair-fed animals.

Table 4. Oxidative Burst and Phagocytosis 30 Days Following PFDA Treatment

TRT	DCF %GRN P < 0.7493	DCF MFI P < 0.9393	DCF + PMA %GRN P < 0.5012	DCF + PMA MFI P < 0.8145	PI.SA. % RED P < 0.3056	PI.SA. MFI P < 0.8546
<i>Ad lib</i> -fed control	46.8 ± 28.3	21.7 ± 5.4	58.1 ± 26.0	25.5 ± 5.4	94.2 ± 6.0	30.6 ± 3.7
PFDA 20 mg/kg	24.8 ± 27.1	20.7 ± 5.4	31.3 ± 28.1	26.2 ± 7.4	77.4 ± 25.6	32.0 ± 4.2
PFDA 50 mg/kg	22.0 ± 33.8	23.7 ± 7.7	29.2 ± 29.5	28.0 ± 7.0	92.5 ± 8.0	28.8 ± 7.0
Pair-fed 20 mg/kg	38.7 ± 33.7	21.2 ± 5.4	57.6 ± 34.3	29.0 ± 6.7	93.8 ± 6.8	30.4 ± 5.0
Pair-fed 50 mg/kg	28.7 ± 26.9	20.5 ± 5.4	54.3 ± 36.5	31.7 ± 11.3	94.6 ± 4.5	30.4 ± 1.7

Fischer 344 rats were treated with 20 mg/kg or 50 mg/kg PFDA 30 days prior to sacrifice. *Ad libitum*-fed and pair-fed controls were included in the study. Peritoneal macrophages were isolated and adherence purified. Cells were incubated with dichlorofluorescein diacetate (DCFHDA) ± phorbol myristate acetate (PMA) ± propidium iodide (PI) labelled *staphylococcus aureus* (SA) for 30 minutes at 37°C. Shown is treatment (TRT) versus mean percent positive (green(GRN) - DCF; red -PI.SA.) and mean fluorescence intensity (MFI). These data represent 4 experiments where cells were combined from 2 genetically identical animals per group. Fluorescence was measured for 10,000 cells per group.

At 30 days following PFDA treatment, an average of 94% of macrophages from *ad libitum*-fed animals ingested PI-labelled bacteria at a mean fluorescence intensity of 31. The mean percentage of cells ingesting bacteria was decreased by 17-18% (n.s.) at a dose of 20 mg/kg PFDA when compared to pair-fed and *ad libitum*-fed controls whereas no significant differences were observed between macrophages isolated from 50 mg/kg PFDA treated rats and pair-fed controls.

c. Prostaglandin E (PGE) Production

1) 8 Day Experiments

LPS-stimulated macrophages from *ad libitum*-fed animals produced an average of 2185 ± 1135 pg/ml PGE (Table 5). The level of PGE produced by macrophages from rats treated with 20 mg/kg PFDA was increased by 78% and 27% (n.s.) respectively when compared to pair-fed and *ad libitum*-fed controls. The level of PGE produced by macrophages from rats treated with 50 mg/kg PFDA was decreased by 7-9% (n.s.) respectively when compared to *ad libitum*-fed and pair-fed controls. The levels of PGE produced by macrophages from pair-fed 20 and 50 animals were decreased by 28% (n.s.) and 2% (n.s.) respectively when compared to *ad libitum*-fed controls.

Table 5. Prostaglandin E (PGE) Production 8 Days After PFDA Treatment

TREATMENT	% BOUND P < 0.7111	CONCENTRATIO N (pg/ml, p < 0.2448)
<i>Ad libitum</i> -fed	8.5 ± 4.9	2185.0 ± 1134.7
PFDA (20 mg/kg)	5.7 ± 5.0	2785.0 ± 1171.5
PFDA (50 mg/kg)	8.5 ± 3.8	1980.0 ± 946.1
20 mg/kg Pair-fed	9.9 ± 2.9	1567.5 ± 916.4
50 mg/kg Pair-fed	7.9 ± 3.8	2130.0 ± 999.8

Fischer 344 rats were treated with 20 mg/kg or 50 mg/kg PFDA 8 days prior to sacrifice. *Ad libitum*-fed and pair-fed controls were included in the study. Peritoneal macrophages were incubated for 24 hours \pm lipopolysaccharide (LPS). Supernatants were filtered and stored at -70°C until analysis. Shown are data from 4 experiments, where duplicate samples and standards were analyzed using a commercial radioimmunoassay for PGE levels.

2) 30 Day Experiments

LPS-stimulated macrophages from *ad libitum*-fed animals produced an average of 1990 ± 1200 pg/ml PGE (Table 6). The mean level of PGE produced by macrophages from PFDA-treated rats was increased by 10% (n.s.) when compared to *ad libitum*-fed controls and by 16% (n.s.) when compared to pair-fed controls at a dose of 20 mg/kg. The mean level of PGE produced by macrophages from 50 mg/kg PFDA-treated rats was decreased by 13% (n.s.) when compared to *ad libitum*-fed controls and by 17% (n.s.) when compared to pair-fed controls.

Table 6. Prostaglandin E (PGE) Production 30 Days After PFDA Treatment

TREATMENT	% BOUND P < 0.8868	CONCENTRATION (pg/ml, p < 0.9195)
<i>Ad libitum</i> -fed	14.3 ± 16.0	1990.0 ± 1200.4
PFDA (20 mg/kg)	7.9 ± 4.1	2195.0 ± 1040.2
PFDA (50 mg/kg)	9.9 ± 3.1	1735.0 ± 685.2
20 mg/kg Pair-fed	10.0 ± 6.4	1895.0 ± 1177.8
50 mg/kg Pair-fed	10.1 ± 8.4	2086.2 ± 1020.8

Fischer 344 rats were treated with 20 mg/kg or 50 mg/kg PFDA 30 days prior to sacrifice. *Ad libitum*-fed and pair-fed controls were included in the study. Peritoneal macrophages were incubated for 24 hours \pm lipopolysaccharide (LPS). Supernatants were filtered and stored at -70°C until analysis. These data represent 4 experiments where duplicate samples and standards were analyzed using a commercial radioimmunoassay for PGE levels.

d. IL-1 Production1) 8 Day Experiments

LPS-stimulated macrophages from *ad libitum*-fed rats produced a mean level of IL-1 of 1.01 ng/ml (Table 7). Macrophages from 20 mg/kg PFDA-treated rats produced 26% (n.s.) more IL-1 in response to LPS when compared to *ad libitum*-fed controls and 21% (n.s.) more when compared to pair-fed controls. Macrophages from 50 mg/kg PFDA-treated rats produced 19% (n.s.) less IL-1 in response to LPS when compared to *ad libitum*-fed controls and 25% (n.s.) less when compared to pair-fed controls. Pair-fed 20 mg/kg and 50 mg/kg rats did not have significant changes in IL-1.

Table 7. IL-1 Production 8 Days After PFDA Treatment

TREATMENT	IL-1 PRODUCTION(ng/ml) p < 0.1651
<i>Ad libitum</i> -fed control	1.01 ± 0.32
PFDA (20 mg/kg)	1.26 ± 0.30
PFDA (50 mg/kg)	0.82 ± 0.30
20 mg/kg Pair-fed	1.04 ± 0.54
50 mg/kg Pair-fed	1.10 ± 0.51

Fischer 344 rats were treated with 20 mg/kg or 50 mg/kg PFDA 8 days prior to sacrifice. *Ad libitum*-fed and pair-fed controls were included in the study. Peritoneal macrophages were incubated for 24 hours ± lipopolysaccharide (LPS). Supernatants were filtered and stored at -70°C until analysis. These supernatants were added to LBRM 331A5 cells (IL-1 dependent, 1×10^5 /ml) and were incubated with a submitogenic concentration of PMA (1:500) for 24 hours at 37°C. Supernatants from these cultures were then incubated with CTLL-2 cells for 24 hours at 37°C. These data represent 4 experiments where [3 H]-thymidine incorporation by CTLL-2 cells was determined for duplicate samples and standards:

2) 30 Day Experiments

Macrophages from *ad libitum*-fed rats produced a mean level of 1.2 ng/ml IL-1 in response to LPS (Table 8). Macrophages from 20 mg/kg PFDA-treated rats produced 21% (n.s.) less IL-1 than *ad libitum*-fed controls but 32% (n.s.) more IL-1 than pair-fed controls. Macrophages from 50 mg/kg PFDA-treated rats produced 17% less IL-1 (n.s.) than *ad libitum*-fed controls but 11% more IL-1 than pair-fed controls. Macrophages from 20 and 50 pair-fed rats produced 40% and 25% less IL-1 respectively than *ad libitum*-fed controls.

e. Peritoneal Cell Recovery Following PFDA Treatment

1) 8 Day Experiments

The average total recovery of peritoneal cells from *ad libitum*-fed animals was 1.9×10^7 cells per animal (Table 9). At 8 days following PFDA treatment, mean peritoneal cell recovery was decreased by 38% (n.s.) following 20 mg/kg PFDA treatment when compared to *ad libitum*-fed controls and by 22% (n.s.) when compared to pair-fed controls. At a dose of 50 mg/kg PFDA a significant reduction of 58% ($p < 0.05$) was observed when compared to *ad libitum*-fed controls and a 47% (n.s.) reduction was observed when compared to pair-fed controls. Peritoneal cells from 20 pair-fed and 50 pair-fed animals were decreased by 5% and 21% respectively when compared to *ad libitum*-fed controls.

Table 8. IL-1 Production 30 Days After PFDA Treatment

TREATMENT	IL-1 PRODUCTION (ng/ml) p < 0.4262
<i>Ad libitum</i> -fed control	1.20 ± 0.79
PFDA (20 mg/kg)	0.95 ± 0.72
PFDA (50 mg/kg)	1.00 ± 0.61
20 mg/kg Pair-fed	0.72 ± 0.29
50 mg/kg Pair-fed	0.90 ± 0.55

Fischer 344 rats were treated with 20 mg/kg or 50 mg/kg PFDA 30 days prior to sacrifice. *Ad libitum*-fed and pair-fed controls were included in the study. Peritoneal macrophages were incubated for 24 hours ± lipopolysaccharide (LPS). Supernatants were filtered and stored at -70°C until analysis. These supernatants were added to LBRM 331A5 cells (IL-1 dependent, 1×10^5 /ml) and were incubated with a submitogenic concentration of PMA (1:500) for 24 hours at 37°C. Supernatants from these cultures were then incubated with CTLL-2 cells for 24 hours at 37°C. These data represent 4 experiments where [3 H]-thymidine incorporation in CTLL-2 cells was determined for duplicate samples and standards.

2) 30 Day Experiments

At 30 days following PFDA treatment, mean peritoneal cell recovery from *ad libitum*-fed controls was 1.5×10^7 cells per animal (Table 9). Animals treated with 20 mg/kg PFDA had an average of 33% (n.s.) fewer peritoneal cells than *ad libitum*-fed and 47% (n.s.) fewer peritoneal cells than pair-fed controls. Animals treated with 50 mg/kg PFDA had a significant reduction in mean peritoneal cell recovery of 87% ($p < 0.05$) when compared to *ad libitum*-fed controls and a reduction of 83% (n.s.) when compared to pair-fed controls. Pair-fed 20 animals had 27% (n.s.) more cells than *ad libitum*-fed controls, whereas pair-fed 50 animals had 20% (n.s.) fewer cells than *ad libitum*-fed controls.

Table 9. Total Peritoneal Cell Number Following PFDA Treatment

TREATMENT	TOTAL PERITONEAL CELL NUMBER AT 8 DAYS FOLLOWING PFDA TREATMENT $p < 0.0481$	TOTAL PERITONEAL CELL NUMBER AT 30 DAYS FOLLOWING PFDA TREATMENT $p < 0.0100$
<i>Ad libitum</i> -fed controls	$1.9 \times 10^7 \pm 7.7 \times 10^6$	$1.5 \times 10^7 \pm 7.4 \times 10^6$
PFDA (20 mg/kg)	$1.4 \times 10^7 \pm 3.1 \times 10^6$	$1.0 \times 10^7 \pm 7.9 \times 10^6$
PFDA (50 mg/kg)	$0.8 \times 10^7^* \pm 3.8 \times 10^6$	$0.2 \times 10^7^* \pm 9.8 \times 10^5$
20 mg/kg Pair-fed	$1.8 \times 10^7 \pm 4.3 \times 10^6$	$1.9 \times 10^7 \pm 3.4 \times 10^6$
50 mg/kg Pair-fed	$1.5 \times 10^7 \pm 3.8 \times 10^6$	$1.2 \times 10^7 \pm 5.7 \times 10^6$

Fischer 344 rats were treated with 20 mg/kg or 50 mg/kg PFDA 8 or 30 days prior to sacrifice. *Ad libitum*-fed and pair-fed controls were included in the study. Cells were isolated and erythrocyte-depleted when necessary, and were counted using trypan blue and a hemocytometer. These data represent cell counts performed on 4 experiments for each treatment period. * = significant difference from *ad libitum*-fed control value, $p < 0.05$ by Tukey's range test.

3. Discussion

The effect of PFDA on innate immunity was studied by measuring NK cytotoxicity and macrophage function. A significant increase in mean NK activity was observed at a dose of 50 mg/kg b.w. PFDA at 30 days but not at 8 days following treatment when compared to *ad libitum*-fed controls. This increase in NK activity appeared to be associated with PFDA-induced anorexia and/or propylene glycol administration because no significant differences in NK activity were observed when compared to pair-fed controls. It is not known if the increase in NK activity is due to an increase in the number of NK cells in the spleen or if it is due to increased activation of the normal resident NK cell population, possibly due to an increase in interferon gamma or interleukin (IL)-2.

PFDA is a peroxisomal proliferator (Van Rafelghem and Andersen, 1985). Although peroxisomal proliferators frequently cause hepatocarcinomas (Rao and Reddy, 1987), PFDA has not been reported to induce hepatocarcinomas, and short-term carcinogenesis tests have been negative for PFDA (Rogers *et al.*, 1982). However, PFDA has been shown to cause oxidative DNA damage in rat liver (Takagi *et al.*, 1991). The increase in NK activity reported here, if sustained for a long period of time, could prevent the establishment of hepatocarcinomas in PFDA-treated rats despite the occurrence of peroxisomal proliferation.

The effect of dietary restriction without malnutrition has been studied in mice.

Restricted rodents live longer (Ross, 1961) and develop spontaneous tumors less often or later in life than unrestricted controls (Tannenbaum, 1942; Ross and Bras, 1965; Ross and Bras, 1973). Dietary restriction without malnutrition has been shown to enhance polyinosinic:polycytidylic acid-induced NK responses in aged mice, but does not alter NK responses without induction (Weindruch *et al.*, 1983). In our system, propylene glycol could be acting as an inducer of NK activity similar to that of polyinosinic:polycytidylic acid. In combination with dietary restriction, this could then result in enhanced NK activity. The rats in our study, however, undergo dietary restriction with malnutrition because diets are not balanced with nutrients adjusted to the level of lowered food intake. The rats in our study may also be stressed due to toxicity and restricted food intake. Stress has also been shown to increase NK cell activity under certain experimental conditions (Jain and Stevenson, 1991).

Macrophage function, another aspect of innate immunity, was also assessed. At 8 days following PFDA treatment, there was a significant decrease in the number of macrophages isolated and a decrease in the function of the remaining macrophages when compared to both *ad libitum*-fed and pair-fed controls. The oxidative burst in response to stimulation by phorbol ester was significantly decreased, and phagocytosis was also decreased although the difference was not statistically significant in these cells when compared to both *ad libitum*-fed and pair-fed animals. At 30 days following PFDA treatment, the effects of PFDA on macrophage number were also statistically significant and effects on percentages of hydrogen peroxide-producing cells in response to PMA were of larger magnitude (although not statistically significant) demonstrating a cumulative effect of PFDA in these cells over time.

PFDA, when injected into the peritoneum of these rats, is probably taken up to an extent by resident macrophages as has been described for perfluorochemical emulsions (Geyer, 1983). PFDA is apparently cytotoxic to these cells, as shown by the decrease in cell number. Other groups have reported PFDA-induced cytotoxicity for different cell types (Levitt and Liss, 1986; Liss *et al.*, 1987). Perfluorodecanoic acid has been shown to dissolve plasma membranes and release intracellular contents (Levitt and Liss, 1986). PFDA also inhibits the ability of remaining cells to perform two important functions, phagocytosis and oxidative burst, which could increase the animal's susceptibility to disease. The increase in the severity of the effect of PFDA over a longer time period suggests that perhaps PFDA accumulates in macrophages altering their function until the levels of PFDA become cytotoxic. Macrophages have been shown to retain most undigested foreign matter, leading to concentration of xenobiotics in their cytoplasm (Lewis and Adams, 1985). Although the decrease in phagocytic function was not statistically significant, it appears to be a dose-dependent response at 8 days following PFDA treatment. The decrease in phagocytic function could be due to disruption of energy metabolism required to drive phagocytosis; it could be due to direct alteration of the cellular membrane, to defective signal transduction through receptors; and/or to decreases in proteins required for actin assembly. Phagocytosis is a complex process that can be divided into two major parts: adherence to specific receptors on the plasma membrane that interact with

molecular components on the surface of the pathogen, and internalization or ingestion of the adherent particle (Sveum *et al.*, 1986). The assembly and organization of actin required for extension of pseudopodia for phagocytosis and cell movement in macrophages is regulated by a number of proteins. These include actin-binding protein and α -actinin which are responsible for filament crosslinking. Gelsolin, severin, and acumentin are involved in the regulation of filament length and assembly. Profilin stabilizes actin monomers (Hartwig, 1986). Two possible pathways for regulating cytoplasmic actin filament structure have been identified: 1. mobilization of calcium, and 2. phosphorylation of proteins by protein kinase C. Both are activated by receptor-ligand binding. Since PFDA is a fatty acid analog, it has been proposed that PFDA incorporates into cellular membranes and alters their function and/or integrity (George and Andersen, 1986; Levitt and Liss, 1986). PFDA may also interact with lipid components of mitochondrial membranes, leading to inhibition of electron transport and uncoupling of electron transport and oxidative phosphorylation (Langley, 1990). PFDA also causes reductions in serum protein and total liver protein (George and Andersen, 1986; Van Rafelghem *et al.*, 1988). In addition, PFDA alters (decreases or induces) various liver proteins (Witzmann and Parker, 1991). Although most of these effects have been determined in hepatocytes, it may be predicted that similar alterations will occur in other cell types, especially macrophages which are capable of taking up perfluorochemicals and retaining them (Lewis and Adams, 1985; Bucala *et al.*, 1983). Weinstock *et al.* (1986) have demonstrated decreased colloidal carbon clearance by Kupffer cells (liver macrophages) in response to perfluorochemical emulsions.

Hydrogen peroxide production is important in microbicidal and tumoricidal activities of macrophages (Badwey and Karnovsky, 1980; Nathan, 1983). A consistent decrease in oxidative burst was observed which was sometimes but not always statistically significant. This could be due to one or more alterations in this complex process. A decrease in PMA receptor number, affinity, or signalling through protein kinase C could be involved (Nathan and Tsunawaki, 1986; Meyers *et al.*, 1985). In addition, changes in glucose transport, NADPH levels, cytochrome b559 content, catalase content, GSH peroxidase or reductase content or myeloperoxidase content could be involved (Nathan and Tsunawaki, 1986; Segal *et al.*, 1983) reflecting membrane alterations by PFDA. As stated previously, PFDA has been shown to alter membrane integrity (George and Andersen, 1986) and electron transport in hepatocytes (Langley, 1990) and it is probable that similar alterations may occur in macrophages. Human recombinant IFN α or β has been shown to decrease capacity of macrophages to secrete reactive oxygen metabolites (Ezekowitz and Gordon, 1986).

Mitogen-induced PGE and IL-1 production were not significantly altered by PFDA treatment. Slight and variable changes in cytokine production were noted depending on dose and time course of PFDA treatment.

Increases in PGE₂ production have been shown to be immunosuppressive (Rappaport and Dodge, 1982). Slight increases in PGE were observed at 8 and 30 days following treatment with PFDA at a dose of 20 mg/kg b.w. when compared to

both *ad libitum*-fed and pair-fed controls. Increased oxidation of fatty acids induced by PFDA has been described which may result in increased prostaglandin production (Fox, 1981). Slight decreases in PGE production were observed at the toxic dose of 50 mg/kg b.w. PFDA at 8 days and 30 days following PFDA treatment when compared to both *ad libitum*-fed and pair-fed controls.

IL-1, alone or through stimulation of IL-2, IL-6, and $\text{TNF}\alpha$ is required for activation of monocytes, B cells, T cells, and neutrophils and promotes hematopoiesis, clotting and phagocytosis. Increases in IL-1 production can lead to PGE_2 production by fibroblasts, macrophages, and endothelial cells (Neckeloff *et al.*, 1991), as well as loss of appetite and fever. Cytokines may also stimulate secretion of glucocorticoids and glucocorticoids can in turn potentiate metabolic effects of cytokines or inhibit their production. In addition, glucocorticoids themselves are known to be immunosuppressive (Paul, 1989). At 8 days following treatment with 20 mg/kg, a slight increase in IL-1 production was observed whereas a decrease in IL-1 production was observed at 30 days following treatment. A consistent but slight decrease in IL-1 production was observed at both 8 and 30 days following treatment with 50 mg/kg PFDA when compared to *ad libitum*-fed and pair-fed controls at 8 days and when compared to *ad libitum*-fed controls at 30 days. The mechanism by which PFDA might alter IL-1 production has not been determined. Possibly, PFDA may downregulate IL-1 receptor expression, IL-1 mRNA, or PFDA may inhibit translation of IL-1.

PFDA may be shifting the balance of cytokine production toward PGE_2 directly or indirectly, thereby creating a microenvironment conducive to immunosuppression. One must consider, however, that these cells have been removed from the *in vivo* microenvironment and the systemic influence of PFDA exposure. If PFDA did not cause permanent changes in cellular processes, data on cytokine production *in vitro* may not reflect what is occurring in the intact PFDA-treated animal. Further experiments measuring levels of cytokines during the induction of an *in vivo* immune response to pathogens or tumors would be helpful to substantiate these findings.

C. MECHANISMS OF ALTERATION OF IMMUNITY BY IN VIVO PFDA TREATMENT

1. Introduction

This chapter presents data on proposed mechanisms of immunomodulation by PFDA. The toxicity of PFDA to thymocytes and splenocytes has been determined. In addition, the ratio of helper:suppressor T cells has been examined. The capacity of lymphocytes to respond to mitogens and immunogen has been determined by the lymphocyte blast transformation (LBT) assay. Functional receptor (IL-2R and MHC Class II) and IL-2 production has been assessed.

Determination of the effect of PFDA on IL-2 production is important because IL-2 plays a central role in the expansion of cells that have been selected by antigen and in the induction of their effector functions. IL-2 is required for the expansion of T cells, NK cells, and B cells, and blocking of IL-2 production interrupts T-cell development (Reviewed by Swain, 1991).

Determination of the effect of PFDA on MHC Class II expression is also important because T lymphocytes recognize complexes of processed antigen which are bound to MHC molecules on the surfaces of primarily macrophages or B cells. Complexes of antigen bound to MHC molecules must reach a critical threshold for T cell activation (Unanue, 1984). The ability of lymphocytes to proliferate in response to antigen and mitogen, as measured by the LBT assay is a critical first step in an adequate immune response.

2. Methods and Materials

a. Preparation of Mononuclear Spleen Cell Cultures

1) Lymphocyte Blast Transformation (LBT)

For each experiment, mononuclear spleen cells (1×10^6 /ml, 100 μ l) were combined from two identically treated, genetically identical animals and were incubated in triplicate 96-well flatbottom microtiter plates (Corning) with LBT medium with or without mitogen [Con A (2 μ g/ml), LPS (10 μ g/ml), or KLH (10 μ g/ml)] for 66 hours at 37°C. [3 H]-thymidine (0.5 μ Ci/well) was added and cultures were incubated for a further 6 hours. Samples were harvested onto filter mats, dried, and incorporation of nucleotide was determined by liquid scintillation counting. Experiments were repeated four times. Data were not averaged prior to analysis.

2) Interleukin-2 (IL-2) Production

Mononuclear spleen cells (2×10^6 /ml, 100 μ l) were incubated in duplicate in 96-well roundbottom microtiter plates (Corning) with LBT medium with or without Con A (2 μ g/ml) for 24 hours at 37°C. Supernatants were isolated and stored at -70°C until analysis. Supernatants were diluted 1:2, 1:4, 1:8 and were incubated with IL-2 dependent CTLL-2 (ATCC TIB 214) cells 1×10^5 /ml, 100 μ l for 24 hours. [3 H]-thymidine (0.5 μ Ci/well) was added for the last 6 hours of culture. Samples were

harvested onto filter mats, dried, and incorporation of nucleotide was determined by liquid scintillation counting. Experiments were repeated four times. Data were not averaged prior to analysis.

3) IL-2 Receptor Expression

Cultures were prepared as described under IL-2 production. Cells were pelleted and identically treated cells from 6 wells were combined into a total volume of 200 μ l. Cells were aliquotted in 100 μ l volumes and were pelleted. Monoclonal anti-rat IL-2 receptor which was FITC conjugated (Serotec) was diluted 1:4 and 10 μ l was added per well. Cells were subjected to brief low speed vortex to resuspend them. Cells were incubated at 4°C for 30 minutes and were washed three times prior to fixation with 2% paraformaldehyde. Duplicate samples (5000 cells each) were analyzed by flow cytometry using an excitation wavelength of 488 nm with a laser power of 200 mW. Filters used to select for different parameters were FALS and FITC green fluorescence (550 long pass dichroic and 525 band pass filters). Experiments were repeated four times. Data were not averaged prior to analysis.

4) Analysis of Cell Markers

i. Major Histocompatibility Complex Antigen Expression

FITC-labelled monoclonal antibody to rat RT1B (class II polymorphic antigen, Serotec) was diluted 1:10 and 1×10^6 erythrocyte-depleted spleen cells were resuspended in 10 μ l of the antibody solution. Cells were incubated at 4°C for 30 minutes and were washed three times prior to fixation with 2% paraformaldehyde. Duplicate samples (10,000 cells) were analyzed by flow cytometry using an excitation wavelength of 488 nm and a laser power of 200 mW. Filters used to select for different parameters were: FALS and FITC green fluorescence (550 long pass and 525 band pass filters). Experiments were repeated four times. Data were not averaged prior to analysis.

ii. CD4/CD8 Populations

Mononuclear spleen or thymus cells (1×10^6) were incubated with 10 μ l FITC-labelled mouse monoclonal anti-rat CD4 (Serotec) and 10 μ l Phycoerythrin (PE)-labelled mouse monoclonal anti-rat CD8 (Serotec) for 30 minutes at 4°C. Cells were washed three times prior to fixation with 2% paraformaldehyde. Duplicate samples (5000 cells) were analyzed by flow cytometry using an excitation wavelength of 488 nm and a laser power of 300 mW. Filters used to select for different parameters were: FALS, FITC green fluorescence (CD4, 550 long pass dichroic and 525 band pass filters) and PE yellow fluorescence (CD8, 600 short pass dichroic and 575 band pass filters). Experiments were repeated four times. Data were not averaged prior to analysis.

iii. B Cell Populations

Mononuclear spleen cells (1×10^6) were incubated with monoclonal anti-rat LCA antigen (present only on B cells, 1:100 dilution, Serotec) for 30 minutes at 4°C and then washed 2 times. Secondary antibody (FITC-labelled Goat anti-mouse IgG, F_c fragment specific, Jackson Laboratories) was added at a 1:2 dilution followed by a further 30 minute incubation. Cells were washed 3 times and were fixed in 2% paraformaldehyde. Duplicate samples were analyzed by flow cytometry using an excitation wavelength of 488 nm and a laser power of 300 mW. Filters used to select for different parameters were: FALS and FITC green fluorescence (550 long pass dichroic and 525 band pass filters). Experiments were repeated four times. Data were not averaged prior to analysis.

3. **Results**

a. Lymphocyte Blast Transformation

1) Response to Con A

i. 8 Day Experiments

At 8 days following PFDA treatment, a significant decrease in mean lymphoproliferation in response to con A was observed at 20 mg/kg PFDA (32%, $p < 0.05$) and a slight decrease was noted at 50 mg/kg PFDA (8%, n.s.) when compared to the *ad libitum*-fed controls (Table 10). A significant decrease of 24% ($p < 0.05$) was observed at 20 mg/kg PFDA when compared to pair-fed controls. A significant increase of 39% was observed at a dose of 50 mg/kg when compared to pair-fed controls. Decreases in mean lymphoproliferation in cells from pair-fed controls were observed when compared to *ad libitum*-fed controls (11%, 20 mg/kg pair-fed, n.s.; 34%, 50 mg/kg pair-fed, $p < 0.05$).

ii. 30 Day Experiments

At 30 days following PFDA treatment, a slight decrease in mean lymphoproliferation was observed at 20 mg/kg PFDA (9%, n.s.) when compared to the *ad libitum*-fed control and a decrease of 47% ($p < 0.05$) was observed when compared to the pair-fed control. An increase in mean lymphoproliferation was observed at 50 mg/kg PFDA (52%, n.s.) when compared to the *ad libitum*-fed control whereas no difference was observed when compared to the pair-fed control. An increase in mean lymphoproliferation was observed for cells from pair-fed animals (74%, $p < 0.05$, 20 mg/kg pair-fed; 59%, n.s., 50 mg/kg pair-fed) (Table 11).

Table 10. Lymphoproliferation 8 Days Following PFDA Treatment

TRT	RESPONSE TO CON A (DPM) (P<0.0001)	RESPONSE TO LPS (DPM) (P<0.3268)	RESPONSE TO KLH (DPM) (P<0.0001)
<i>Ad libitum</i> -fed control	270277 ± 58649	21751 ± 18723	10460 ± 9644
PFDA (20 mg/kg)	183026 ^{a,b} ± 89393	23900 ± 8165	11346 ^b ± 8070
PFDA (50 mg/kg)	248388 ^b ± 64460	24856 ± 10965	7470 ± 6565
20 mg/kg Pair-fed	241670 ± 81916	28560 ± 18739	19174 ^a ± 22235
50 mg/kg Pair-fed	178830 ± 101919	25109 ± 12467	8951 ± 6844

Fischer 344 rats were treated with 20 mg/kg or 50 mg/kg PFDA 8 days prior to sacrifice. *Ad libitum*-fed and pair-fed controls were included in the study. Erythrocyte-depleted spleen cells (1×10^6 /ml) were incubated ± Con A or LPS or KLH for 66 hours at 37°C. ^3H -thymidine (0.5 μCi /well) was added for the remaining 6 hours of incubation. Samples were harvested and incorporation of radiolabel into triplicate cultures was determined. These data represent mean disintegrations per minute (DPM) ± s.d. for 4 experiments. (a) significantly different from *ad libitum*-fed controls, (b) significantly different from pair-fed controls, $p < 0.05$.

2) Response to LPS

i. 8 Day Experiments

Slight increases in mean lymphoproliferation in response to LPS were observed with all treatment groups at 8 days following PFDA treatment when compared to the *ad libitum*-fed controls (10%, 20 mg/kg, n.s.; 14%, 50 mg/kg, n.s.; 31%, 20 mg/kg pair-fed, n.s.; 15.4%, 50 mg/kg pair-fed, n.s.). When compared to the pair-fed control, PFDA at a dose of 20 mg/kg decreased lymphoproliferation by 16% (n.s.), whereas PFDA at a dose of 50 mg/kg did not alter lymphoproliferation when compared to the pair-fed control (n.s., Table 10).

ii. 30 Day Experiments

At 30 days following PFDA treatment, a decrease in mean lymphoproliferation was observed at 20 mg/kg PFDA when compared to the *ad libitum*-fed controls (34%, n.s.) whereas no change in mean lymphoproliferation was observed at 50 mg/kg or with cells from the 20 mg/kg pair-fed and 50 mg/kg pair-fed animals. When compared

to pair-fed controls, PFDA (20 mg/kg) decreased proliferation by 36% (n.s.) whereas PFDA (50 mg/kg) did not alter proliferation (Table 11).

3) Response to KLH

i. 8 Day Experiments

A slight increase in mean lymphoproliferation in response to KLH was observed at 20 mg/kg PFDA when compared to the *ad libitum*-fed controls (8%, n.s.) whereas a significant decrease was observed when compared to the pair-fed control (41%, $p < 0.05$) at 8 days following treatment. A decrease in mean lymphoproliferation was observed at 50 mg/kg (29%, n.s.) when compared to *ad libitum*-fed controls and when compared to pair-fed controls (16%, n.s., Table 10). An increase in mean lymphoproliferation was observed for cells from 20 pair-fed animals when compared to the *ad libitum*-fed controls (83.3%, $p < 0.05$), whereas a slight decrease in mean lymphoproliferation was observed for cells from 50 pair-fed animals (14.4%, n.s.).

Table 11. Lymphoproliferation 30 Days Following PFDA Treatment

TRT	RESPONSE TO CON A (DPM) ($P < 0.0021$)	RESPONSE TO LPS (DPM) ($P < 0.6393$)	RESPONSE TO KLH (DPM) ($P < 0.3616$)
<i>Ad libitum</i> - fed control	146146 \pm 57086	33617 \pm 25379	14819 \pm 10935
PFDA (20 MG/KG)	133209 ^b \pm 43967	22286 \pm 20333	10784 \pm 8998
PFDA (50 MG/KG)	221658 \pm 125309	34165 \pm 20979	17721 \pm 22593
20 mg/kg PAIR-FED	253730 ^a \pm 117356	34691 \pm 32470	24167 \pm 23870
50 mg/kg PAIR-FED	232135 \pm 124384	34870 \pm 31630	18251 \pm 21723

Fischer 344 rats were treated with 20 mg/kg or 50 mg/kg PFDA 30 days prior to sacrifice. *Ad libitum*-fed and pair-fed controls were included in the study. Erythrocyte-depleted spleen cells (1×10^6 /ml) were incubated \pm Con A or LPS or KLH for 66 hours at 37°C. [³H]-thymidine (0.5 μ Ci/well) was added for the remaining 6 hours of incubation. Samples were harvested and incorporation of radiolabel into triplicate cultures was determined. These data represent mean disintegrations per minute (DPM) \pm s.d. for 4 experiments.

ii. 30 Day Experiments

A decrease in mean lymphoproliferation in response to KLH was observed at 20 mg/kg when compared to the *ad libitum*-fed controls (27%, n.s.) and pair-fed controls (55%, n.s.), whereas an increase in mean lymphoproliferation in response to KLH was observed at 30 days following PFDA treatment for the remainder of the treatments when compared to the *ad libitum*-fed controls (20%, 50 mg/kg, n.s.; 63%, 20 mg/kg pair-fed, n.s.; 23%, 50 mg/kg pair-fed, n.s.) No change was observed in lymphoproliferation between 50 mg/kg PFDA treatment and pair-fed controls (Table 11).

b. Interleukin-2 (IL-2) Production

1) 8 Day Experiments

Mean levels of IL-2 produced by Con A stimulated cells from PFDA-treated rats were decreased by 8% at 20 mg/kg and increased by 23% at 50 mg/kg when compared to *ad libitum*-fed controls. Mean levels of IL-2 produced by cells obtained from 20 and 50 pair-fed animals were decreased by 41% and 67% respectively when compared to *ad libitum*-fed controls (Table 12).

Table 12. Interleukin-2 Production 8 Days Following PFDA Treatment

TREATMENT	IL-2 PRODUCTION (DPM 1:4 DILUTION) P < 0.0001
<i>Ad libitum</i> -fed control	20126 ± 8444
PFDA (20 mg/kg)	18481 ± 10870
PFDA (50 mg/kg)	24846 ^b ± 11770
20 mg/kg Pair-fed	11770 ± 8351
50 mg/kg Pair-fed	6681 ^a ± 4423

Fischer 344 rats were treated with 20 mg/kg or 50 mg/kg PFDA 8 days prior to sacrifice. *Ad libitum*-fed and pair-fed controls were included in the study. Erythrocyte-depleted spleen cell samples (2×10^6 /ml) were incubated ± Con A for 24 hours at 37°C. Supernatants were assayed for IL-2 levels by incubation with duplicate CTLL-2 (IL-2 dependent) cell cultures and determining [³H]-thymidine incorporation. These data represent mean disintegrations per minute (DPM) ± s.d. for 4 experiments. (a) significantly different from *ad libitum*-fed controls, (b) significantly different from pair-fed controls.

2) 30 Day Experiments

Mean levels of IL-2 produced by Con A stimulated cells from PFDA-treated rats were decreased by 9% (n.s.) and 27% (n.s.) at a dose of 20 mg/kg b.w. when compared to *ad libitum*-fed and pair-fed controls respectively. Mean IL-2 production

was increased in PFDA-treated rats by 85% (n.s.) and 11% (n.s.) for 50 mg/kg b.w. doses respectively when compared to *ad libitum*-fed and pair-fed controls. Mean levels of IL-2 produced by Con A stimulated cells from 20 and 50 pair-fed rats were increased by 25% (n.s.) and 67% (n.s.) respectively when compared to *ad libitum*-fed controls (Table 13).

Table 13. Interleukin-2 Production 30 Days Following PFDA Treatment

TREATMENT	IL-2 PRODUCTION (DPM 1:4 DILUTION) P<0.0168
<i>Ad libitum</i> -fed control	17213 ± 11114
PFDA (20 mg/kg)	15660* ± 13435
PFDA (50 mg/kg)	31853 ± 12412
20 Pair-fed	21588 ± 16844
50 Pair-fed	28777 ± 18598

Fischer 344 rats were treated with 20 mg/kg or 50 mg/kg PFDA 30 days prior to sacrifice. *Ad libitum*-fed and pair-fed controls were included in the study. Erythrocyte-depleted spleen cell samples (2×10^6 /ml) were incubated \pm Con A for 24 hours at 37°C. Supernatants were assayed for IL-2 levels by incubation with duplicate CTLL-2 (IL-2 dependent) cell cultures and determining [3 H]-thymidine incorporation. These data represent mean disintegrations per minute (DPM) \pm s.d. for 4 experiments. * = significantly different from the 50 mg/kg PFDA treatment group, $p < 0.05$ by Tukey's range test.

c. Interleukin-2 Receptor (IL-2R) Expression

1) 8 Day Experiments

An average of 24% of resting spleen cells from *ad libitum*-fed controls expressed IL-2R (Table 13). Fewer resting spleen cells expressed IL-2R (20% and 4%, n.s.) following 20 mg/kg PFDA treatment when compared to pair-fed and *ad libitum*-fed controls respectively. An increase of 17% (n.s.) of spleen cells expressing IL-2R was observed following 50 mg/kg PFDA treatment when compared to *ad libitum*-fed controls, but a decrease of 4% (n.s.) was observed when compared to pair-fed controls. Increases in cells expressing IL-2R of 21% and 23% were observed in spleen cells from 20 pair-fed and 50 pair-fed rats respectively when compared to *ad libitum*-fed controls.

Following stimulation with Con A, approximately 53% of cells from *ad libitum*-fed control rats expressed IL-2R. Very slight differences (less than 13%) were observed when comparing IL-2R expression in spleen cells from PFDA-treated rats to that of pair-fed rats and *ad libitum*-fed controls.

Table 14. IL-2 Receptor (R) Expression 8 Days Following PFDA Treatment

TREATMENT	% RESTING CELLS WHICH EXPRESS IL-2R (P < 0.4693)	% CON A STIMULATED CELLS WHICH EXPRESS IL-2R (P < 0.8799)
<i>Ad libitum</i> -fed control	24.5 ± 6.8	53.0 ± 19.1
PFDA (20 mg/kg)	23.6 ± 5.7	51.5 ± 19.8
PFDA (50 mg/kg)	28.8 ± 10.0	53.7 ± 16.3
20 mg/kg Pair-fed	29.6 ± 11.0	59.3 ± 22.7
50 mg/kg Pair-fed	30.1 ± 10.4	59.3 ± 17.8

Fischer 344 rats were treated with 20 mg/kg or 50 mg/kg PFDA 8 days prior to sacrifice. *Ad libitum*-fed and pair-fed controls were included in the study. Erythrocyte depleted spleen cell samples (2×10^6 /ml) were incubated \pm Con A for 24 hours at 37°C. Duplicate samples were incubated with FITC-labelled monoclonal anti-rat IL-2R for 30 minutes at 4°C, were washed and fixed with 2% paraformaldehyde prior to analysis by flow cytometry. These data represent mean percent IL-2R positive cells \pm s.d. for 4 experiments.

2) 30 Day Experiments

An average of 37% of resting spleen cells from *ad libitum*-fed controls expressed IL-2R (Table 14). Decreases of 17% (n.s.) and 5% (n.s.) in IL-2R expression were observed in resting spleen cells from 20 mg/kg PFDA-treated rats when compared to pair-fed and *ad libitum*-fed controls respectively. Increases of 21% (n.s.) and 57% (n.s.) in IL-2R expression were observed in resting spleen cells from 50 mg/kg PFDA-treated rats when compared to pair-fed and *ad libitum*-fed controls respectively. Following stimulation with Con A, an average of 66% of cells isolated from *ad libitum*-fed control rats expressed IL-2R. Changes in the level of IL-2R were less than 6% (n.s.) in Con A stimulated cells from rats treated with 20 mg/kg PFDA when compared to pair-fed and *ad libitum*-fed controls. Increases of 19% (n.s.) and 14% (n.s.) in IL-2 expression were observed in Con A stimulated cells from rats treated with 50 mg/kg PFDA when compared to pair-fed and *ad libitum*-fed controls (n.s.). Increases of 5-6% (n.s.) of Con A stimulated cells expressing IL-2R was observed amongst cells isolated from 20 and 50 pair-fed animals respectively when compared to *ad libitum*-fed controls.

Table 15. IL-2 Receptor (R) Expression 30 Days Following PFDA Treatment

TREATMENT	% RESTING CELLS WHICH EXPRESS IL-2R (P < 0.2858)	% CON A STIMULATED CELLS WHICH EXPRESS IL-2R (P < 0.1227)
<i>Ad libitum</i> -fed control	36.6 ± 23.2	66.3 ± 15.6
PFDA (20 mg/kg)	38.6 ± 20.4	65.8 ± 10.9
PFDA (50 mg/kg)	57.4 ± 12.8	79.1 ± 5.2
20 mg/kg Pair-fed	46.7 ± 18.3	69.7 ± 10.0
50 mg/kg Pair-fed	47.6 ± 24.9	70.1 ± 9.3

Fischer 344 rats were treated with 20 mg/kg or 50 mg/kg PFDA 30 days prior to sacrifice. *Ad libitum*-fed and pair-fed controls were included in the study. Erythrocyte depleted spleen cell samples (2×10^6 /ml) were incubated \pm Con A for 24 hours at 37°C. Duplicate samples were incubated with FITC-labelled monoclonal anti-rat IL-2R for 30 minutes at 4°C, were washed and fixed with 2% paraformaldehyde prior to analysis by flow cytometry. These data represent mean percent IL-2R positive cells \pm s.d. for 4 experiments.

d. Major Histocompatibility Complex II (MHC II) Antigen Expression

1) 8 Day Experiments

MHC Class II antigen was expressed on an average of 41% of spleen cells isolated from *ad libitum*-fed controls (Table 16). The percentage of cells expressing MHCII was reduced following PFDA treatment by 17% (n.s.) at a dose of 20 mg/kg when compared to both pair-fed and *ad libitum*-fed controls. MHC II expression was reduced by 17% (n.s.) and 21% (n.s.) at a dose of 50 mg/kg, when compared to pair-fed and *ad libitum*-fed controls respectively. Little or no change was observed in MHC II expression amongst pair-fed rats when compared to *ad libitum*-fed controls. The density of receptors per cell was similar through all treatments as indicated by mean fluorescence intensities

2) 30 Day Experiments

MHC Class II antigen was expressed on an average of 37% of spleen cells isolated from *ad libitum*-fed controls (Table 17). At 30 days following PFDA treatment, minute changes in MHC II expression were observed. Decreases in MHC II expression of 17% (n.s.) and 9% (n.s.) were observed at 20 mg/kg when compared to pair-fed and *ad libitum*-fed controls respectively. Decreases of 12% (n.s.) and 3% (n.s.) in MHC II expression were observed at 50 mg/kg when compared to pair-fed and *ad libitum*-fed controls respectively. Increases of 10-11% (n.s.) in MHC II

expression were noted amongst pair-fed animals when compared to *ad libitum*-fed controls. The density of receptors per cell was similar through all treatments as indicated by mean fluorescence intensities (105-107).

Table 16. MHC II Expression 8 Days Following PFDA Treatment

TREATMENT	% CELLS WHICH EXPRESS MHC II (P < 0.4897)	MEAN FLUORESCENCE INTENSITY (P < 0.6749)
<i>Ad libitum</i> -fed control	41.0 ± 9.0	116.2 ± 9.4
PFDA (20 mg/kg)	34.1 ± 12.5	115.1 ± 7.6
PFDA (50 mg/kg)	32.3 ± 13.4	113.5 ± 6.0
20 mg/kg Pair-fed	41.0 ± 13.8	109.9 ± 13.8
50 mg/kg Pair-fed	39.0 ± 11.6	116.0 ± 8.1

Fischer 344 rats were treated with 20 mg/kg PFDA or 50 mg/kg PFDA 8 days prior to sacrifice. *Ad libitum*-fed and pair-fed controls were included in the study. Duplicate erythrocyte-depleted spleen cell samples (1×10^6) were incubated with FITC-labelled monoclonal anti-rat RT1B for 30 minutes at 4°C, were washed and fixed with 2% paraformaldehyde prior to analysis by flow cytometry. These data represent mean percent MHCII positive cells ± s.d. for 4 experiments.

e. CD4/CD8 Expression

1) Spleen

i. 8 Day Experiments

At 8 days post PFDA treatment, splenocytes from 20 mg/kg treated rats had insignificant changes in CD4+ cells (less than 6%); slight increases in CD8+ cells (20% & 4%, n.s.); and decreases in double positive cells (17% & 3%, n.s.) when compared to pair-fed and *ad libitum*-fed controls respectively (Table 18). Cells from 50 mg/kg treated rats had insignificant changes in CD4+ cells (less than 4%); decreases in CD8+ cells (8% & 16.1%, n.s.); and increases in double positive cells (62% & 43%, n.s.) when compared to pair-fed and *ad libitum*-fed controls. Splenocytes from 20 mg/kg pair-fed and 50 mg/kg pair-fed rats were insignificantly enriched in CD4+ cells by less than 7%, and slightly depleted in CD8+ cells (14% & 9% respectively) when compared to the *ad libitum*-fed controls. Splenocytes from pair-fed 20 rats had increased double positive cells (17.3%, n.s.) when compared to the *ad libitum*-fed controls whereas splenocytes from pair-fed 50 rats had decreased double positive cells (12%) when compared to the *ad libitum*-fed controls.

Table 17. MHC II Expression 30 Days Following PFDA Treatment

TREATMENT	% CELLS WHICH EXPRESS MHC II (P < 0.3128)	MEAN FLUORESCENCE INTENSITY
<i>Ad libitum</i> -fed control	36.9 ± 7.7	107.3 ± 11.7
PFDA (20 mg/kg)	33.6 ± 6.8	106.8 ± 11.2
PFDA (50 mg/kg)	35.7 ± 7.6	107.3 ± 8.1
20 mg/kg Pair-fed	40.7 ± 10.1	106.8 ± 12.9
50 mg/kg Pair-fed	40.8 ± 11.9	105.5 ± 11.1

Fischer 344 rats were treated with 20 mg/kg PFDA or 50 mg/kg PFDA 30 days prior to sacrifice. *Ad libitum*-fed and pair-fed controls were included in the study. Duplicate erythrocyte-depleted spleen cell samples (1×10^6) were incubated with FITC-labelled monoclonal anti-rat RT1B for 30 minutes at 4°C, were washed and fixed with 2% paraformaldehyde prior to analysis by flow cytometry. These data represent mean percent MHCII positive cells ± s.d. for 4 experiments.

Table 18. CD4/CD8 Expression in the Spleen 8 Days Following PFDA Treatment

TREATMENT	% CD4 + SPLEEN CELLS (P < 0.9364)	% CD8 + SPLEEN CELLS (P < 0.0603)	% DP SPLEEN CELLS (P < 0.6486)
<i>Ad libitum</i> -fed control	32.8 ± 6.5	34.2 ± 6.8	7.5 ± 6.5
PFDA (20 mg/kg)	34.6 ± 4.0	35.5 ± 5.5	7.3 ± 5.6
PFDA (50 mg/kg)	34.1 ± 5.4	28.7 ± 5.2	10.7 ± 6.1
20 mg/kg Pair-fed	33.9 ± 4.4	29.5 ± 4.3	8.8 ± 6.0
50 mg/kg Pair-fed	35.1 ± 8.1	31.2 ± 4.4	6.6 ± 4.7

Fischer 344 rats were treated with 20 mg/kg PFDA or 50 mg/kg PFDA 8 days prior to sacrifice. *Ad libitum*-fed and pair-fed controls were included in the study. Duplicate erythrocyte-depleted spleen cell samples (1×10^6) were incubated with FITC-labelled monoclonal anti-rat CD4 and phycoerythrin (MwE)-labelled monoclonal anti-rat CD8 for 30 minutes at 4°C, were washed and fixed with 2% paraformaldehyde prior to analysis by flow cytometry. These data represent mean percent CD4⁺/CD8⁺ cells ± s.d. for 4 experiments.

ii. 30 Day Experiments

At 30 days following PFDA treatment, all treatment groups had insignificantly increased percentage of CD4 + cells when compared to the *ad libitum*-fed controls (4-13%, n.s., Table 19). Differences in CD4⁺ cells between PFDA-treated and pair-fed controls were less than 5% (n.s.) Increases in the percentage of CD8 + cells were also observed among all treatment groups when compared to the *ad libitum*-fed controls (9%, 20 mg/kg; 6%, 50 mg/kg; 22%, 20 pair-fed; 1%, 50 pair-fed, n.s.). Differences in CD8 + cells between PFDA-treated and pair-fed controls were less than 11% (n.s.). Decreases in the percentage of double positive cells were also observed when compared to the *ad libitum*-fed controls (30.6% 20 mg/kg; 8.2%, 50 mg/kg, 16.3%, 20 pair-fed; 18.4%, 50 pair-fed, n.s.). The percentage of double positive cells was decreased by 17% at 20 mg/kg and increased by 12% when compared to pair-fed controls.

Table 19. CD4/CD8 Expression in the Spleen 30 Days Following PFDA Treatment

TREATMENT	% CD4 + SPLEEN CELLS (P<0.9537)	% CD8 + SPLEEN CELLS (P<0.8259)	% DP SPLEEN CELLS (P<0.6890)
<i>Ad libitum</i> - fed control	32.7 ± 10.2	26.6 ± 7.6	4.9 ± 3.6
PFDA (20 mg/kg)	34.0 ± 13.0	29.0 ± 11.8	3.4 ± 1.6
PFDA (50 mg/kg)	37.0 ± 13.8	28.1 ± 9.3	4.5 ± 2.0
20 mg/kg Pair-fed	35.7 ± 11.0	32.4 ± 14.1	4.1 ± 1.3
50 mg/kg Pair-fed	36.3 ± 15.7	26.9 ± 12.2	4.0 ± 1.1

Fischer 344 rats were treated with 20 mg/kg PFDA or 50 mg/kg PFDA 30 days prior to sacrifice. *Ad libitum*-fed and pair-fed controls were included in the study. Duplicate erythrocyte-depleted spleen cell samples (1×10^6) were incubated with FITC-labelled monoclonal anti-rat CD4 and phycoerythrin (PE)-labelled monoclonal anti-rat CD8 for 30 minutes at 4°C, were washed and fixed with 2% paraformaldehyde prior to analysis by flow cytometry. These data represent mean percent CD4⁺/CD8⁺ cells ± s.d. for 4 experiments.

2) Thymusi. 8 Day Experiments

At 8 days following PFDA treatment, a 24% (n.s.) decrease in the percentage of CD4+ cells and a 3% decrease in the percentage of CD8+ cells (n.s) was observed at 20 mg/kg PFDA when compared to the *ad libitum*-fed controls, whereas no change was noted in the double positive (DP) population, and an increase of 11% was observed in the double negative (DN) population when compared to the *ad libitum*-fed controls (Table 20). When compared to pair-fed controls, 20 mg/kg PFDA treatment did not alter CD4,

Table 20. CD4/CD8 Expression in the Thymus 8 Days Following PFDA Treatment

TRT	% CD4 + IN THYMUS P<0.2573	%CD8 + IN THYMUS P<0.803 2	% DP IN THYMUS P<0.0017	% DN IN THYMUS P<0.438 3
<i>Ad libitum</i> - fed control	17.3 ± 5.5	11.4 ± 4.6	63.6 ±5.7	10.8 ± 8.5
PFDA (20 mg/kg)	13.1 ± 4.1	11.1 ± 3.4	63.7 ± 4.4	12.0 ± 11.1
PFDA (50 mg/kg)	19.7 ± 12.8	12.9 ± 8.1	39.0 ^a ± 19.3	28.8 ± 33.9
20 Pair-fed	13.0 ± 6.6	10.1 ± 4.4	60.3 ± 10.2	16.6 ± 18.6
50 Pair-fed	13.4 ± 6.6	10.0 ± 5.3	53.8 ± 17.5	22.8 ± 28.8

Fischer 344 rats were treated with 20 mg/kg PFDA or 50 mg/kg PFDA 8 days prior to sacrifice. *Ad libitum*-fed and pair-fed controls were included in the study. Duplicate thymus cell samples (1×10^6) were incubated with FITC-labelled monoclonal anti-rat CD4 and phycoerythrin (PE)-labelled monoclonal anti-rat CD8 for 30 minutes at 4°C, were washed and fixed with 2% paraformaldehyde prior to analysis by flow cytometry. These data represent mean CD4⁺/CD8⁺ cells ± s.d. for 4 experiments. (a) significantly different from *ad libitum*-fed controls.

CD8, or DP expression in the thymus by more than 10% (n.s.). DN expression was decreased by 28% (n.s.) at this dose, however, when compared to the pair-fed control. Thymocytes from 50 mg/kg PFDA-treated animals had increases in CD4 +

expression of 47% (n.s.) and 14% (n.s.) and increases in CD8 + expression of 29% (n.s.) and 13% (n.s.), with a marked decrease of 27% (n.s.) and 39% ($p < 0.05$) in the double positive population, and a sharp increase of 26% (n.s.) and 162% (n.s.) in the double negative population (162% n.s.) when compared to the pair-fed and *ad libitum*-fed controls respectively. Thymocytes from pair-fed 20 and 50 animals had decreased percentages of all cell populations except the double negative population. The CD4 + population was decreased by 25% (n.s.) and 22% (n.s.) respectively, the CD8 + population was decreased by 11% (n.s.) and 12% (n.s.) respectively; and the double positive population decreased by 5% (n.s.) and 15% (n.s.) respectively; the double negative population was increased by 54% (n.s.) and 111% respectively (n.s.) when compared to the *ad libitum*-fed controls.

ii. 30 Day Experiments

At 30 days following PFDA treatment, increases of 20% (n.s.) and 8% (n.s.) were observed in CD4 + expression, and increases of 56% and 13% were observed in DN expression at 20 mg/kg when compared to *ad libitum*-fed and pair-fed controls respectively (Table 21). Changes in CD8 + expression were less than 11% (n.s.) when compared to *ad libitum*-fed and pair-fed controls. Thymocytes from 50 mg/kg PFDA-treated rats demonstrated decreases in CD4 + (12% & 9%, n.s.) and DP cells (9% & 14%, n.s.) when compared to pair-fed and *ad libitum*-fed controls respectively.

Table 21. CD4/CD8 Expression in the Thymus 30 Days Following PFDA Treatment

TREATMENT	% CD4 + IN THYMUS P < 0.7197	% CD8 + IN THYMUS P < 0.9838	% DP IN THYMUS P < 0.1794	% DN IN THYMUS P < 0.2918
<i>Ad libitum</i> fed control	8.5 ± 3.6	14.5 ± 6.6	68.9 ± 5.7	6.1 ± 4.2
PFDA (20 mg /kg)	10.2 ± 5.3	16.1 ± 5.3	62.6 ± 6.0	9.5 ± 6.4
PFDA (50 mg/kg)	7.7 ± 2.6	16.1 ± 10.6	59.4 ± 11.3	15.4 ± 16.9
20 mg/kg Pair-fed	9.4 ± 2.6	16.6 ± 7.9	64.2 ± 7.8	8.4 ± 5.3
50 mg/kg Pair-fed	8.8 ± 3.5	15.7 ± 8.0	65.3 ± 7.6	8.8 ± 5.8

Fischer 344 rats were treated with 20 mg/kg PFDA or 50 mg/kg PFDA 30 days prior to sacrifice. *Ad libitum*-fed and pair-fed controls were included in the study. Duplicate thymus cell samples (1×10^6) were incubated with FITC-labelled monoclonal anti-rat CD4 and phycoerythrin (PE)-labelled monoclonal anti-rat CD8 for 30 minutes at 4°C, were washed and fixed with 2% paraformaldehyde prior to analysis by flow cytometry. These data represent mean percent CD4 + /CD8 + cells ± s.d. for 4 experiments.

Changes in CD8 + expression were less than 11% (n.s.) when compared to pair-fed and *ad libitum*-fed controls. Increases in DN cells of 75% (n.s.) and 152% (n.s.) when

compared to pair-fed and *ad libitum*-fed controls. Thymocytes from pair-fed 20 mg/kg and 50 mg/kg rats had slight increases in CD4+ cells of 11% (n.s.) and 3% (n.s.), in CD8+ cells of 14% (n.s.) and 8% (n.s.), and in double negative cells of 38% (n.s.) and 44% (n.s.) respectively, whereas the double positive population was altered by less than 7% (n.s.) when compared to *ad libitum*-fed controls.

f. B Cell Populations

1) 8 Day Experiments

At 8 days following PFDA treatment, the mean percentage of B cells in spleens from 20 mg/kg or 50 mg/kg PFDA-treated rats did not differ by more than 8% (n.s.) when compared to *ad libitum*-fed controls. When compared to pair-fed controls, PFDA at a dose of 20 mg/kg decreased the mean percentage of B cells in the spleen by 9% (n.s.) whereas a dose of 50 mg/kg PFDA increased the mean percentage of B cells by 26%. Pair-fed rats (20 mg/kg) had an increase of 19% in the percentage of B cells when compared to the *ad libitum*-fed controls, whereas a decrease of 19% was noted for B cells from 50 mg/kg pair-fed rats when compared to the *ad libitum*-fed controls (Table 22).

Table 22. Percentage of B Cells in the Spleen 8 Days Following PFDA Treatment

TREATMENT	% B CELLS IN SPLEEN (P < 0.1266)
<i>Ad libitum</i> -fed control	28.2 ± 6.6
PFDA (20 mg/kg)	30.4 ± 7.1
PFDA (50 mg/kg)	28.7 ± 7.4
20 mg/kg Pair-fed	33.5 ± 8.1
50 mg/kg Pair-fed	22.8 ± 5.4

Fischer 344 rats were treated with 20 mg/kg PFDA or 50 mg/kg PFDA 8 days prior to sacrifice. *Ad libitum*-fed and pair-fed controls were included in the study. Duplicate spleen cell samples (1×10^6) were incubated with monoclonal anti-rat LCA (present only on B cells) for 30 minutes at 4°C, were washed and incubated with FITC-labelled goat anti-mouse IgG₁ (F_c fragment specific) for a further 30 minutes at 4°C. Cells were then washed and fixed with 2% paraformaldehyde prior to analysis by flow cytometry. These data represent mean percent LCA+ cells ± s.d. for 4 experiments.

2) 30 Day Experiments

At 30 days following PFDA treatment, a decrease of 11% (n.s.) in the mean percentage of B cells in the spleen was observed at a dose of 20 mg/kg. PFDA treatment at a dose of 20 mg/kg increased mean B cell levels in the spleen by 20% (n.s.) when compared to pair-fed controls. Pair-fed 20 mg/kg and 50 mg/kg pair-fed rats had decreased percentages of 26% and 12% when compared to *ad libitum*-

fed controls. An increase in the percentage of B cells of 42% ($p < 0.05$) and 25% (n.s.) was observed at a dose of 50 mg/kg PFDA-treated rats when compared to the *ad libitum*-fed and pair-fed controls respectively (Table 23).

Table 23. Percentage of B Cells in the Spleen 30 Days Following PFDA Treatment

TREATMENT	% B CELLS IN SPLEEN ($P < 0.0125$)
<i>Ad libitum</i> -fed control	25.4 ± 6.7
PFDA (20 mg/kg)	22.6 ± 7.7
PFDA (50 mg/kg)	$31.7^* \pm 7.7$
20 mg/kg Pair-fed	18.8 ± 7.8
50 mg/kg Pair-fed	22.3 ± 5.3

Fischer 344 rats were treated with 20 mg/kg PFDA or 50 mg/kg PFDA 30 days prior to sacrifice. *Ad libitum*-fed and pair-fed controls were included in the study. Duplicate spleen cell samples (1×10^6) were incubated with monoclonal anti-rat LCA (present only on B cells) for 30 minutes at 4°C, were washed and incubated with FITC-labelled goat anti-mouse IgG₁ (F_c fragment specific) for a further 30 minutes at 4°C. Cells were then washed and fixed with 2% paraformaldehyde prior to analysis by flow cytometry. These data represent mean percent LCA⁺ cells \pm s.d. for 4 experiments. * = significantly different from 20 mg/kg pair-fed controls.

g. Peripheral Blood Leukocyte Count and Differential

1) 8 Day Experiments

Mean values for *ad libitum*-fed controls for neutrophil, lymphocyte, and monocyte counts were $1.5 \times 10^9/l$, $6.5 \times 10^9/l$, and $0.3 \times 10^9/l$ respectively (Table 24). Decreases in neutrophil counts of 13% and 7% were observed with 20 mg/kg and 50 mg/kg PFDA treatment respectively when compared to *ad libitum*-fed controls. No change in neutrophil counts was observed between 20 mg/kg PFDA-treated rats and pair-fed controls, whereas an increase of 75% (n.s.) in neutrophil counts was observed in 50 mg/kg PFDA-treated rats when compared to pair-fed controls. Decreases in neutrophil counts of 13% and 47% were observed amongst corresponding pair-fed animals when compared to *ad libitum*-fed controls.

Decreases in lymphocyte counts of 8% and 25% were observed with 20 mg/kg and 50 mg/kg PFDA treatment respectively when compared to *ad libitum*-fed controls. Lymphocyte counts of PFDA-treated rats at both 20 mg/kg and 50 mg/kg were increased by 22% (n.s.) when compared to pair-fed controls. Decreases in lymphocyte counts of 25% and 38% ($p < 0.05$) were observed amongst corresponding pair-fed animals.

All treatment groups had 67% fewer peripheral blood monocytes than *ad libitum*-fed controls. No difference was observed between monocyte counts of 20 mg/kg PFDA-treated animals and pair-fed controls, whereas monocyte counts were

doubled in 50 mg/kg PFDA-treated rats when compared to pair-fed controls.

Table 24. Peripheral Blood Leukocyte Counts 8 Days Following PFDA Treatment

TREATMENT	NEUT. X 10 ⁹ /L P < 0.3941	LYMPH. X 10 ⁹ /L P < 0.0199	MONO. X 10 ⁹ /L P < 0.6418
<i>ad libitum</i> -fed control	1.5 ± 0.2	6.5 ± 1.2	0.3 ± 0.1
PFDA (20 mg/kg)	1.3 ± 0.7	6.0 ± 1.5	0.2 ± 0.1
PFDA (50 mg/kg)	1.4 ± 0.5	4.9 ± 1.3	0.2 ± 0.1
20 mg/kg Pair-fed	1.3 ± 0.6	4.9 ± 0.8	0.2 ± 0.3
50 mg/kg Pair-fed	0.8 ± 0.4	4.0 ^a ± 1.0	0.1 ± 0.05

Blood samples were collected by cardiac puncture following anesthetization 8 days following PFDA treatment and were transferred to EDTA-treated tubes. Duplicate samples were analyzed in the clinical hematology department at the Ohio State University Veterinary Teaching Hospital. Shown are mean neutrophil, lymphocyte, and monocyte counts ± s.d. for 4 experiments. (a) significantly different from *ad libitum*-fed controls, $p < 0.05$.

Table 25. Peripheral Blood Leukocyte Counts 30 Days Following PFDA Treatment

TREATMENT	NEUT. X 10 ⁹ /L P < 0.0009	LYMPH. X 10 ⁹ /L P < 0.4909	MONO. X 10 ⁹ /L P < 0.3091
<i>ad libitum</i> -fed control	1.1 ± 0.6	5.1 ± 2.0	0.1 ± 0.1
PFDA (20 mg/kg)	1.2 ± 0.4	5.3 ± 1.5	0.2 ± 0.1
PFDA (50 mg/kg)	2.7 ^{a,b} ± 0.9	4.2 ± 2.3	0.2 ± 0.2
20 mg/kg Pair-fed	1.3 ± 0.8	4.0 ± 1.1	0.1 ± 0.04
50 mg/kg Pair-fed	1.6 ± 0.3	4.1 ± 1.6	0.2 ± 0.2

Blood samples were collected following anesthetization by cardiac puncture 30 days following PFDA treatment and were transferred to EDTA-treated tubes. Duplicate samples were analyzed in the clinical hematology department at the Ohio State University Veterinary Teaching Hospital. Shown are mean neutrophil, monocyte, and lymphocyte counts ± s.d. for 4 experiments. a = significantly different from *ad libitum*-fed controls, and b = significantly different from pair-fed controls.

2) 30 Day Experiments

Mean values for neutrophil, lymphocyte, and monocyte counts were $1.1 \times 10^9/l$, $5.1 \times 10^9/l$, and $0.1 \times 10^9/l$ respectively (Table 25). At 30 days following PFDA treatment, neutrophil counts were altered by less than 10% at a dose of 20 mg/kg when compared to *ad libitum*-fed or pair-fed animals. Neutrophil counts were increased following PFDA treatment at a dose of 50 mg/kg by 69% ($p < 0.05$) and 145% ($p < 0.05$) when compared to pair-fed and *ad libitum*-fed controls respectively. Increases in neutrophil counts of 18% (n.s.) and 45% (n.s.) were observed amongst pair-fed animals when compared to *ad libitum*-fed controls.

Increases of 32% and 4% in lymphocyte count were observed following 20 mg/kg PFDA treatment when compared to pair-fed and *ad libitum*-fed controls respectively, and a decrease of 18% (n.s.) was observed following 50 mg/kg PFDA treatment when compared to *ad libitum*-fed controls, whereas no change was observed when compared to pair-fed controls. Decreases of 20-22% were observed amongst pair-fed animals when compared to *ad libitum*-fed controls.

Monocyte counts were doubled amongst 20 mg/kg PFDA-treated animals when compared to pair-fed and *ad libitum*-fed controls. Monocyte counts were also doubled amongst 50 mg/kg PFDA treated animals and 50 pair-fed animals when compared to *ad libitum*-fed controls, but were unchanged amongst 20 pair-fed animals when compared to *ad libitum*-fed controls.

h. Total Cell Numbers - Thymus and Spleen

i. 8 Day Experiments

Following removal of erythrocytes from spleen cell preparations an average of 2.1×10^8 cells were isolated per spleen from *ad libitum*-fed control animals. Decreases in cell number by 11% (n.s.) and 24% (n.s.) were observed at a dose of 20 mg/kg when compared to pair-fed and *ad libitum*-fed controls respectively. Decreases in cell number of 61% (n.s.) and 76% (n.s.) were observed following PFDA treatment at a dose of 50 mg/kg when compared to pair-fed and *ad libitum*-fed controls respectively (Table 26). Decreases in cell number by 14% and 38% were observed in spleens isolated from corresponding pair-fed animals.

An average of 4.6×10^8 cells were isolated per thymus from *ad libitum*-fed control animals (Table 27). Decreases in cell number by 35% (n.s.) and 39% (n.s.) were observed following treatment with PFDA at a dose of 20 mg/kg when compared to pair-fed and *ad libitum*-fed controls respectively. Decreases in thymocyte number of 85% ($p < 0.05$) and 87% ($p < 0.05$) were observed following PFDA treatment at a dose of 50 mg/kg when compared to pair-fed and *ad libitum*-fed controls respectively. Decreases in cell number by 6% (n.s.) and 13% (n.s.) were observed amongst thymocytes isolated from corresponding pair-fed animals when compared to *ad libitum*-fed controls.

Table 26. Total Cell Numbers 8 Days Following PFDA Treatment

TREATMENT	TOTAL SPLEEN CELL NUMBER (P<0.2160)	TOTAL THYMUS CELL NUMBER (P<0.0092)
<i>Ad libitum</i> -fed control	$2.1 \times 10^8 \pm 1.2 \times 10^8$	$4.6 \times 10^8 \pm 2.3 \times 10^8$
PFDA (20 mg/kg)	$1.6 \times 10^8 \pm 0.96 \times 10^8$	$2.8 \times 10^8 \pm 1.0 \times 10^8$
PFDA (50 mg/kg)	$0.5 \times 10^8 \pm 0.44 \times 10^8$	$0.61 \times 10^{8a,b} \pm 0.49 \times 10^8$
20 mg/kg Pair-fed	$1.8 \times 10^8 \pm 1.0 \times 10^8$	$4.3 \times 10^8 \pm 1.5 \times 10^8$
50 mg/kg Pair-fed	$1.3 \times 10^8 \pm 0.91 \times 10^8$	$4.0 \times 10^8 \pm 1.4 \times 10^8$

Fischer 344 rats were treated with 20 mg/kg or 50 mg/kg PFDA 8 days prior to sacrifice. *Ad libitum*-fed and pair-fed controls were included in the study. Spleen and thymus single cell suspensions were prepared, erythrocyte-depleted when necessary and were counted using trypan blue and a hemocytometer. Shown are mean cell counts \pm s.d. for 4 experiments. (a) significantly different from *ad libitum*-fed controls, (b) significantly different from pair-fed controls.

Table 27. Total Cell Numbers 30 Days Following PFDA Treatment

TREATMENT	TOTAL SPLEEN CELL NUMBER (P<0.2019)	TOTAL THYMUS CELL NUMBER (P>0.05)
<i>Ad libitum</i> -fed control	$2.1 \times 10^8 \pm 1.2 \times 10^8$	$4.3 \times 10^8 \pm 2.8 \times 10^8$
PFDA (20 mg/kg)	$2.4 \times 10^8 \pm 1.8 \times 10^8$	$4.2 \times 10^8 \pm 2.2 \times 10^8$
PFDA (50 mg/kg)	$0.9 \times 10^8 \pm 0.06 \times 10^8$	$0.4 \times 10^8 \pm 0.35 \times 10^8$
20 mg/kg Pair-fed	$1.8 \times 10^8 \pm 1.1 \times 10^8$	$4.6 \times 10^8 \pm 2.4 \times 10^8$
50 mg/kg Pair-fed	$0.9 \times 10^8 \pm 0.18 \times 10^8$	$2.3 \times 10^8 \pm 1.2 \times 10^8$

Fischer 344 rats were treated with 20 mg/kg or 50 mg/kg PFDA 30 days prior to sacrifice. *Ad libitum*-fed and pair-fed controls were included in the study. Spleen and thymus single cell suspensions were prepared, erythrocyte-depleted when necessary and were counted using trypan blue and a hemocytometer. Shown are mean cell counts \pm s.d. for 4 experiments.

ii. 30 Day Experiments

Average isolated cell number per spleen of *ad libitum*-fed controls was 2.1×10^8 cells (Table 26). Increases in splenocyte number of 33% (n.s.) and 14% (n.s.) were measured 30 days following PFDA treatment at a dose of 20 mg/kg when compared to pair-fed and *ad libitum*-fed controls respectively. Splenocyte counts were decreased by 57% (n.s.) for 50 mg/kg PFDA-treated animals when compared to *ad libitum*-fed controls, whereas mean splenocyte counts amongst 50 mg/kg PFDA-

treated animals were similar to pair-fed control values. Decreases of 14% (n.s.) and 57% (n.s.) were measured for spleens isolated from corresponding pair-fed animals.

An average of 4.3×10^8 cells were isolated per thymus of *ad libitum*-fed controls (Table 27). 30 days following PFDA treatment, thymocyte numbers were decreased by less than 9% (n.s.) at a dose of 20 mg/kg when compared to *ad libitum*-fed and pair-fed controls. Thymocyte numbers were decreased by 83% (n.s.) and 91% (n.s.) following treatment with PFDA at a dose of 50 mg/kg when compared to pair-fed and *ad libitum*-fed controls respectively. An increase of 7% was determined for thymuses isolated from 20 pair-fed animals whereas a decrease of 46% was determined for thymuses isolated from 50 pair-fed animals when compared to *ad libitum*-fed controls.

3. Discussion

This study examined possible mechanisms of PFDA-induced immunomodulation. One of the most important events which occurs during a primary immune response is the proliferation of clones of antigen-specific cells. Because the number of antigen-specific T and B cells is low in lymphoid tissue, the expansion of the pool of antigen reactive effector cells and generation of memory cells is critical for the expression of a specific immune response and for the development of long-lived specific immunity (Paul, 1989). Therefore, the effect of PFDA on immunogen-specific proliferation was examined. In addition, the effects of PFDA on polyclonal mitogen-induced T and B cell activation were examined to bypass required stages of antigen processing and presentation by macrophages and B cells for T cell activation. Lymphoproliferation was rarely significantly different in cells isolated from PFDA-treated rats when compared to *ad libitum*-fed or pair-fed controls. Certain trends were observed, however. Responses to immunogen and mitogens in cells from PFDA-treated rats were reduced in the majority of cases when compared to pair-fed controls.

Decreased responsiveness to immunogen could be due to defective antigen processing and presentation in association with MHC Class II markers and/or defective signal transduction across T cell receptors or surface IgM receptors on B cells. Decreased responsiveness to both immunogens and mitogens could be due to defective IL-2 receptor expression and /or production of IL-2, and/or decreased percentages of CD4+ helper cells (Paul, 1989).

Many of these mechanisms have been evaluated in this study. Antigen-induced T cell proliferation is regulated primarily through the actions of IL-2 on its specific cell surface receptor (Smith, 1986). IL-2 production in response to Con A was significantly greater at 8 days following 50 mg/kg PFDA treatment than that of the pair-fed control. IL-2 production was also elevated, but not significantly so, 30 days following PFDA treatment at that dose. IL-2 production 8 days following PFDA treatment at 20 mg/kg was greater than that of the pair-fed control, whereas at 30 days following PFDA treatment at 20 mg/kg, IL-2 production was reduced when compared to the pair-fed controls. IL-2 receptor expression in response to Con A was not significantly altered following PFDA treatment, but a trend of reduced IL-2R

expression was observed for PFDA-treated cells when compared to pair-fed controls in the majority of cases. The exception was at 30 days, where PFDA, at a dose of 50 mg/kg caused a slight increase in IL-2R expression relative to pair-fed controls. The defects in lymphoproliferation in response to Con A and immunogen might be explained by a reduction in responsiveness to IL-2. The IL-2R may be functionally defective, or the signal transduction pathway across the IL-2R may be defective. This could be tested in future by determining responsiveness of PFDA-treated cells to exogenous IL-2.

Up-regulation of Class II MHC molecules or antigen-presenting cells is required for potent T cell responses to antigen. This is mediated by IL-4 and IFN γ . The regulation of MHC II expression is achieved by a combination of transcriptional and post-transcriptional effects. Lack of Class II expression leads to immunodeficiency, whereas overexpression may lead to autoimmunity (Kara and Glimcher, 1991). PFDA did not cause a significant change in MHC II expression, but there was a trend of reduced MHC II expression at both doses, over both time periods when compared to both pair-fed and *ad libitum*-fed controls. This could impair immunogen-induced T cell responsiveness.

The relative proportion of helper:suppressor cells T cells (CD4:CD8) in lymphoid tissues could have an impact on immune responsiveness. CD4+ cells are required for IL-2 production and clonal expansion, as well as isotype switching (Burstein and Abbas, 1991). Subclasses of CD8+ T cells are responsible for inhibiting lymphocyte proliferation and lymphokine production (Sussman and Wodee, 1991). The balance of CD4+:CD8+ cells does not appear to play a role in the observed PFDA-induced immunosuppression since usually both markers are altered simultaneously and in a small way.

The double positive thymocyte appears to be the target of PFDA-induced cytotoxicity in the thymus. This would probably deplete some of the repertoire of antigens to which the animal could respond and thus may have a deleterious effect on the function of the immune system. In the spleen, T cells are the targets of cytotoxicity as discussed previously, and PFDA does not appear to be selective for either CD4+ or CD8+ cells.

Percentages of B cells might be expected to decrease in the spleen of PFDA-treated rats if PFDA were cytotoxic to them. The percentage of B cells was increased at 50 mg/kg 8 and 30 days following PFDA treatment relative to both pair-fed and *ad libitum*-fed controls. This may reflect decreases in the percentages of T cells and macrophages in the spleen.

The leukocyte cell counts reflect some of the changes noted in the immune system. At 8 days following PFDA treatment, decreases in lymphocyte and monocyte counts were observed relative to *ad libitum*-fed controls. At 30 days following PFDA treatment, lymphocyte counts recovered in 20 mg/kg treated animals, and also improved in 50 mg/kg treated animals relative to *ad libitum*-fed controls. Peripheral blood monocyte counts were also higher, but evidently these monocytes are not trafficking to the peritoneum to become resident macrophages as indicated by total peritoneal cell counts.

One must consider effects of PFDA on the immune system which may be due to direct cytotoxicity. PFDA has been shown to decrease total spleen and thymus cell numbers in a dose-dependent manner.

D. IN VITRO EFFECTS OF PFDA ON IMMUNE SYSTEM PARAMETERS

1. Introduction

In this study, concentrations of PFDA have been selected which are in a range which would be achieved in the body fluids with in vivo doses of 20 mg/kg and 50 mg/kg b.w. PFDA. PFDA does not undergo in vivo metabolism to a more toxic product, and therefore in vitro treatment with PFDA may be expected to cause similar immunomodulation.

Although in vivo immunotoxicity testing can never be entirely replaced, the future trend will be to increase in vitro drug testing in order to decrease required animal numbers. It must first be established, however, that in vitro drug treatment provides as sensitive an indication of immunotoxicity as does in vivo treatment.

2. Methods

a. Immunization and Selection of Effective Concentration Range

Fischer 344 rats were immunized with KLH as described above. Rats were sacrificed and mononuclear cell cultures were prepared. PFDA (0.05g) was dissolved in 400 μ l propylene glycol and diluted in media to a volume of 20 ml. The concentration range of PFDA for in vitro experiments was determined in the LBT assay. The final % propylene glycol added per well was 0.1%. Concentrations below which viability was not altered by PFDA were selected (100, 80, 60, 40, 20 μ M). These concentrations are within the range of in vivo doses of 20 mg/kg and 50 mg/kg based on the assumption of an equal distribution of fluid over 60% of body weight. The 150 μ M concentration of PFDA was included which decreased viability to demonstrate the upper limit of PFDA which could be used. PFDA was added at these concentrations for the duration of the incubation period for each assay. Controls of 0.1% propylene glycol and 0% PFDA/propylene glycol were included.

b. LBT Assay

As previously described except cells were incubated with PFDA (0-150 μ M) for the duration of the assay (72 hours, Ch. III Materials and Methods). Cultures were prepared in triplicate per treatment group and experiments were repeated four times. Data were not averaged prior to analysis.

c. Natural Killer Cell Activity

The assay is the same as described previously except that cells were incubated with PFDA (0-150 μ M) for the duration of the cytotoxicity assay (4 hours, Ch. II Materials and Methods). Cultures were prepared in triplicate per treatment group and experiments were repeated four times. Data were not averaged prior to analysis.

d. IL-2 Production and Receptor Expression

As previously described except cells were incubated with PFDA (0-150 μ M) for

the duration of the culture period (24 hours, Ch. III Materials and Methods). Cells and cell cultures were analyzed in duplicate per treatment group and experiments were repeated four times. Data were not averaged prior to analysis.

e. MHC Expression

As previously described except cells were incubated with PFDA (0-150 μ M) overnight at 37°C (Chapter III Materials and Methods). Cells were analyzed in duplicate per treatment group and experiments were repeated four times. Data were not averaged prior to analysis.

f. Phagocytosis, Oxidative Burst, IL-1 Production, and PGE Production

As previously described except cells were incubated 24 hours with PFDA (0-150 μ M, Chapter III Materials and Methods). Samples were analyzed in duplicate per treatment group and per method of activation. Experiments were repeated four times. Data were not averaged prior to analysis.

3. Results

a. LBT Assay

1) Response to Con A

A concentration dependent decrease in lymphoproliferation was observed with *in vitro* PFDA treatment which ranged from 7% at 20 μ M (n.s.) to 65.9% at 150 μ M ($p < 0.05$) when compared to the control containing no PFDA and no propylene glycol (0 control) (Table 28). Similar decreases were observed when compared to the 0.1% propylene glycol control. Viability was decreased at 15Mw μ M PFDA concentrations.

2) Response to LPS

Lympholiferation decreased from 20-85% of the 0 control in a dose dependent manner with increasing concentrations of PFDA (20-150 μ M) (Table 28). At 100 and 150 μ M differences were statistically significant. Lymphoproliferation was decreased with propylene glycol alone by only 2% of the 0 control.

3) Response to KLH

Lymphoproliferation was decreased from 6-91% (n.s.) of the 0 control at concentrations of PFDA ranging from 20-150 μ M (Table 28). Lymphoproliferation was decreased with propylene glycol alone by 14% of the 0 control. When compared to the propylene glycol control, lymphoproliferation was decreased only at doses of 80 μ M to 150 μ M (6-90%).

Table 28. Lymphoproliferation with in vitro PFDA Treatment

Treatment (Average % Viability)	Response to Con A (CPM) p < 0.0001	Response to LPS (CPM) p < 0.0293	Response to KLH (CPM) p < 0.2723
150 μ M PFDA (71.5 \pm 5.2)	45,634.5* \pm 60954.8	1189.0* \pm 1521.1	2762.6 \pm 4155.0
100 μ M PFDA (87.7 \pm 8.1)	106,279.5 \pm 65450.4	4643.2* \pm 5663.5	21703.5 \pm 28391.8
80 μ M PFDA (90.7 \pm 5.0)	120,875.8 \pm 72212.5	5086.7 \pm 5567.7	26244.0 \pm 34420.2
60 μ M PFDA (90.7 \pm 5.4)	113,483.0 \pm 65694.8	6345.1 \pm 8234.8	30624.3 \pm 28967.2
40 μ M PFDA (90.7 \pm 2.5)	119,527.8 \pm 69719.9	6739.4 \pm 7685.7	27717.7 \pm 38184.0
20 μ M PFDA (92.2 \pm 4.5)	124,165.0 \pm 63824.0	6543.5 \pm 6911.6	27242.1 \pm 34945.0
0.1% Propylene Glycol (90.7 \pm 3.0)	129,532.7 \pm 64631.8	8020.5 \pm 6937.1	27912.4 \pm 32999.3
0% Propylene Glycol/0% PFDA (90.7 \pm 7.6)	133,784.2 \pm 63,846.0	8205.8 \pm 6774.6	32541.4 \pm 39680.1

Splenocytes (1×10^6 /ml) were incubated \pm mitogen (Con A, LPS, or KLH) and PFDA (0 - 150 μ M) for 66 hours at 37°C. [3 H]-thymidine (0.5 μ Ci/well) was added and cultures were incubated for 6 additional hours. Samples were harvested onto filter mats, dried and counted. Shown is treatment versus response, n = 4.

* = significantly different from 0 control, p < 0.05.

b. NK Cell Activity

At 20 μ M PFDA and from 80-150 μ M PFDA, slight decreases in NK activity are observed at all ratios (4.8-12.7%, n.s., 100:1 ratio) whereas slight increases in NK activity are observed at 40-60 μ M PFDA (6-7%, n.s, Table 29) when compared to both the 0 control and the propylene glycol control. No significant changes in viability of YAC-1 or effector cells were noted upon incubation with PFDA.

c. IL-2 Production

IL-2 production was reduced in a dose-dependent manner in PFDA-treated cultures by 11-34% (n.s.) at concentrations of 20-100 μ M PFDA when compared to

both the 0 control and the propylene glycol control (Table 30). IL-2 production was significantly reduced at the toxic dose of 150 μ M PFDA by 69% ($p < 0.05$). Treatment with propylene glycol alone caused a 2% reduction in IL-2 production.

Table 29. Natural Killer Cell Activity with *in vitro* PFDA Treatment

TREATMENT	% DEAD YAC $p < 0.9391$	% DEAD SPLEEN $p < 0.9975$	% DEAD 100:1 E:T $p < 0.7996$	% DEAD 50:1 E:T $P < 0.3289$	% DEAD 25:1 E:T $p < 0.3360$
150 μ M PFDA	7.0 \pm 3.5	10.3 \pm 6.0	34.3 \pm 10.8	29.9 \pm 7.1	20.0 \pm 8.2
100 μ M PFDA	4.8 \pm 2.0	9.3 \pm 8.7	37.4 \pm 13.8	27.5 \pm 8.8	19.8 \pm 7.9
80 μ M PFDA	5.1 \pm 2.4	11.3 \pm 7.3	35.7 \pm 14.5	27.3 \pm 8.8	19.3 \pm 6.2
60 μ M PFDA	4.8 \pm 3.1	12.3 \pm 7.4	41.7 \pm 16.4	36.0 \pm 13.7	24.0 \pm 10.8
40 μ M PFDA	4.8 \pm 2.4	10.2 \pm 5.0	42.0 \pm 12.9	34.2 \pm 13.2	26.8 \pm 13.0
20 μ M PFDA	4.8 \pm 2.4	11.2 \pm 6.7	36.8 \pm 9.0	27.6 \pm 9.8	19.2 \pm 6.7
0.1% PG	4.3 \pm 2.6	11.6 \pm 6.1	37.4 \pm 10.9	30.2 \pm 9.6	19.7 \pm 6.7
0% PG/PFDA	5.3 \pm 2.4	9.5 \pm 4.5	39.3 \pm 12.7	30.2 \pm 9.6	20.8 \pm 6.5

Nonadherent splenocytes (effectors) were incubated with PKH-1 (green fluorescent viable membrane dye) stained target cells (YAC-1) at 100:1, 50:1, and 25:1 ratios \pm PFDA (0-150 μ M, prepared in propylene glycol (PG), 0.1%) in roundbottomed microtiter plates following conjugation for 4 hours at 37°C. Control cultures containing target cells alone or effector cells alone were included. Propidium iodide was added at the end of the culture period and the percentage of dead cells was determined by red fluorescence using flow cytometry.

Table 30. IL-2 Production with in vitro PFDA Treatment

TREATMENT	IL-2 PRODUCTION (CPM OF CTLL-2 CELLS AT 1:4 DILUTION OF SAMPLE) $p < 0.0004$
150 μ M PFDA	1714.0 * \pm 1397.7
100 μ M PFDA	3698.2 \pm 1866.4
80 μ M PFDA	4696.9 \pm 1663.7
60 μ M PFDA	4439.4 \pm 1080.8
40 μ M PFDA	4788.2 \pm 2166.0
20 μ M PFDA	4842.5 \pm 1408.3
0.1% PG	5451.1 \pm 968.6
0% PG/PFDA	5580.2 \pm 1408.5

Splenocytes (2×10^6 /ml) were incubated with Con A for 24 hours at $37^\circ\text{C} \pm$ PFDA (0 - 150 μ M, in propylene glycol (PG), 0.1%). Supernatants were harvested, filtered, and frozen until analysis. Supernatants were analyzed for IL-2 content by proliferation of the IL-2 dependent CTLL-2 cell line as determined by [^3H]-thymidine incorporation.

d. IL-2R Expression

An average of 37% of resting spleen cells expressed IL-2R. IL-2R expression in resting cells was increased by 18-19% (n.s.) at 40-60 μ M PFDA (Table 31). Upon stimulation with Con A, 68.4% of spleen cells expressed IL-2R. IL-2R expression in cells stimulated with con A was altered only slightly (0-7%).

e. MHC Expression

Slight increases (10-20%, n.s.) in the percentage of cells expressing MHC II were observed upon in vitro treatment with PFDA when compared to the 0 control and the propylene glycol control (Table 32). Very slight increases (1-4%, n.s.) in receptor density per cell were observed upon in vitro PFDA treatment at 60 - 150 μ M as determined by mean fluorescence intensity.

Table 31. IL-2 Receptor Expression with in vitro PFDA Treatment

TREATMENT	% RESTING IL-2R ⁺ CELLS p < 0.9643	% IL-2R ⁺ CELLS + CON A p < 0.8891
150 μ M PFDA	36.0 \pm 23.2	67.8 \pm 11.1
100 μ M PFDA	36.9 \pm 25.3	64.8 \pm 14.2
80 μ M PFDA	36.5 \pm 25.5	66.5 \pm 13.0
60 μ M PFDA	43.5 \pm 23.7	73.0 \pm 11.1
40 μ M PFDA	43.9 \pm 24.0	66.7 \pm 15.3
20 μ M PFDA	33.8 \pm 23.8	65.7 \pm 13.3
0.1% PG	36.3 \pm 26.0	67.6 \pm 14.1
0% PG/PFDA	37.0 \pm 24.5	68.4 \pm 13.4

Splenocytes (2×10^6 /ml) were incubated with Con A for 24 hours at $37^\circ\text{C} \pm$ PFDA (0 -150 μ M, in propylene glycol (PG), 0.1%). Cells were then incubated with anti-rat IL-2R antibody for 30 minutes at 4°C . Samples were washed and fixed in 2% paraformaldehyde prior to analysis by flow cytometry.

Table 32. MHC II Expression Following in vitro PFDA Treatment

TREATMENT	% MHC II POSITIVE p < 0.5824	MEAN FLUORESCENCE INTENSITY P < 0.4653
150 μ M PFDA	53.0 \pm 10.3	127.7 \pm 6.3
100 μ M PFDA	48.5 \pm 7.7	124.4 \pm 8.4
80 μ M PFDA	50.3 \pm 11.2	124.2 \pm 7.9
60 μ M PFDA	50.5 \pm 13.2	125.6 \pm 7.4
40 μ M PFDA	54.4 \pm 13.8	123.3 \pm 8.4
20 μ M PFDA	49.3 \pm 12.5	122.6 \pm 6.3
0.1 % PG	44.4 \pm 14.0	119.6 \pm 5.2
0% PG/PFDA	44.1 \pm 17.4	122.6 \pm 10.3

Splenocytes (1×10^6) were incubated \pm PFDA (0 -150 μ M, in propylene glycol (PG), 0.1%) for 24 hours at 37°C . Cells were then incubated with anti-rat RT1B antibody for 30 minutes at 4°C . Samples were washed and fixed in 2% paraformaldehyde prior to analysis by flow cytometry.

f. Phagocytosis and Oxidative Burst

The mean percentage of cells in the 0 control which produced low levels of hydrogen peroxide prior to stimulation (DCF alone) was 78.9% at a mean fluorescence intensity of 21.5. In vitro treatment with PFDA altered the percentage of hydrogen peroxide-producing cells by less than 4% when compared to the 0 control and to the propylene glycol control (n.s., Table 33). Only at the highest levels of PFDA (100-150 μ M) did the mean fluorescence intensity increase by greater than 10% (n.s.) of the control value.

Upon stimulation with PMA, the mean percentage of 0 control cells producing hydrogen peroxide was 81.1 (DCF + PMA) at a mean fluorescence intensity of 34.2. In vitro PFDA treatment caused these values to shift by less than 10% (n.s.) when compared to the 0 control and the propylene glycol control. The mean percentage of 0 control cells which ingested bacteria was 94.1% [PI.SA., % (G + R) + % R]. Of these cells, 32% had an associated oxidative burst. Whereas 92-95% of cells treated with propylene glycol and/or PFDA ingested bacteria, the oxidative burst in response to bacterial ingestion was reduced in some cases by 14-20% of the 0 control (n.s.). PFDA-treated cells had slightly elevated (n.s.) oxidative burst in response to bacterial ingestion when compared to the propylene glycol control.

g. IL-1 Production

Zero control peritoneal macrophages, stimulated with LPS, produced an average of 0.73 ng/ml IL-1 (Table 34). Upon treatment with propylene glycol, a 14% increase in IL-1 production was observed. At 20-40 μ M PFDA, further increases in IL-1 production of 18% (n.s.) and 90% ($p < 0.05$) respectively were observed.

h. PGE Production

Peritoneal macrophages stimulated with LPS produced 1112.6 pg/ml PGE (Table 35). In vitro treatment with propylene glycol and/or PFDA did not alter PGE production by greater than 7% (n.s.) when compared to both the 0 control and the propylene glycol control.

Table 33. Phagocytosis and Oxidative Burst Following in vitro PFDA Treatment

TRT	DCF ALONE % Green p < 0.8960	DCF ALONE MFI p < 0.1423	DCF + PMA % Green p < 0.2230	DCF + PMA MFI p < 0.7131	DCF + PI.SA % G+R p < 0.6837	DCF + PISA % Red p < 0.8707
150 μ M PFDA	75.9 \pm 7.4	25.7 \pm 3.8	81.0 \pm 5.0	32.5 \pm 2.5	33.4 \pm 11.0	58.6 \pm 10.5
100 μ M PFDA	78.0 \pm 7.5	23.7 \pm 4.6	80.5 \pm 6.6	31.2 \pm 2.2	27.9 \pm 4.7	65.8 \pm 4.1
80 μ M PFDA	77.2 \pm 3.6	21.0 \pm 2.9	85.3 \pm 7.2	31.2 \pm 3.1	25.9 \pm 11.2	68.4 \pm 10.1
60 μ M PFDA	78.9 \pm 4.7	22.2 \pm 3.1	86.4 \pm 4.1	31.5 \pm 1.9	27.1 \pm 0.8	67.0 \pm 1.9
40 μ M PFDA	78.8 \pm 5.4	20.7 \pm 2.1	87.0 \pm 4.0	31.7 \pm 4.0	26.6 \pm 9.3	68.2 \pm 9.1
20 μ M PFDA	77.0 \pm 1.5	20.0 \pm 0	85.9 \pm 4.8	31.7 \pm 3.0	33.6 \pm 4.6	60.8 \pm 3.4
0.1% PG	80.6 \pm 2.3	22.0 \pm 1.4	89.4 \pm 4.6	34.0 \pm 3.7	25.8 \pm 11.3	69.5 \pm 11.2
0%PG/PFDA	78.9 \pm 5.3	21.5 \pm 1.3	81.1 \pm 6.2	34.2 \pm 2.9	32.3 \pm 6.4	61.8 \pm 5.4

Resident peritoneal macrophages were isolated and adherence purified. Cells were incubated with PFDA for 24 hours at 37°C. Cells were removed from tissue culture plates using cold cRPMI and scraping. Cells were incubated with dichlorofluorescein diacetate (DCFHDA) \pm PMA \pm PI-labelled staphylococcus aureus (PISA). Cells were analyzed for green fluorescence (DCF) and for red fluorescence (ingestion of PISA) by flow cytometry. Shown is treatment versus percentage of green cells, red + green cells, red cells, and associated mean fluorescence intensities (MFI).

Table 34. IL-1 Production with in vitro PFDA Treatment

TREATMENT	IL-1 PRODUCTION (ng/ml) $p < 0.0001$
150 μ M PFDA	0.70 ± 0.29
100 μ M PFDA	0.69 ± 0.12
80 μ M PFDA	0.76 ± 0.25
60 μ M PFDA	0.74 ± 0.21
40 μ M PFDA	$1.39^* \pm 0.65$
20 μ M PFDA	0.86 ± 0.37
0.1% PG	0.83 ± 0.27
0% PG/PFDA	0.73 ± 0.21

Resident peritoneal macrophages were isolated and adherence purified. Cells were incubated \pm LPS \pm PFDA (0-150 μ M, in 0.1% propylene glycol, PG) for 24 hours at 37°C. Supernatants were harvested and incubated with LBRM331A5 cells which produce IL-2 in the presence of IL-1. IL-2 levels were then estimated by [3 H]-thymidine incorporation in the IL-2 dependent CTLL-2 cell line. * = Significantly different from 0 control, $p < 0.05$ by Tukey's Range Test.

Table 35. Prostaglandin E (PGE) Production with in vitro PFDA Treatment

TREATMENT	% BOUND $p < 1.0$	CONCENTRATION (pg/ml)
150 μ M PFDA	39.9 ± 41.6	1112.6 ± 1232.8
100 μ M PFDA	42.8 ± 42.7	978.5 ± 1065.1
80 μ M PFDA	40.8 ± 41.1	1179.4 ± 1352.0
60 μ M PFDA	44.2 ± 43.4	1209.5 ± 1440.0
40 μ M PFDA	41.9 ± 40.9	1214.1 ± 1474.4
20 μ M PFDA	43.9 ± 42.3	1465.2 ± 1579.4
0.1% PG	43.9 ± 41.1	1403.2 ± 1579.4
0% PG/PFDA	43.1 ± 41.0	1345.1 ± 1616.7

Resident peritoneal macrophages were isolated and adherence purified. Cells were incubated \pm LPS \pm PFDA (0-150 μ M, in 0.1% propylene glycol, PG) for 24 hours at 37°C. Supernatants were harvested and assayed for PGE levels by a commercial radioimmunoassay.

4. Discussion

These studies were done to determine if in vitro immunotoxicity studies could accurately reflect in vivo experiments. Although not statistically significant except at the toxic dose of 150 μ M, consistent dose-dependent decreases in lymphoproliferation to all mitogens were observed when cells were treated in vitro with PFDA. This corresponded to the majority of in vivo lymphoproliferative data. Decreases in lymphoproliferation corresponded to a decrease in IL-2 production. A slightly higher resting IL-2R expression was observed, but IL-2R expression in stimulated cells was normal with PFDA treatment. Insignificant elevations of MHC II antigen expression were observed, and no significant trends in IL-1 and PGE levels were observed. Because IL-2R expression was normal or slightly elevated and IL-2 production was decreased, this may indicate a defect in signal transduction through the IL-2 receptor. Differences in the effect of PFDA on IL-2 production in vivo versus in vitro were observed, but for in vivo and in vitro PFDA treatment a defect in signal transduction through the IL-2 receptor may be indicated.

Changes in NK activity with in vitro PFDA treatment are small (5-13%) and variable, and are not statistically significant. This coincides with the theory that changes observed with in vivo PFDA treatment were probably due to the effects of anorexia and as such will not be mimicked by in vitro PFDA treatment.

In vitro PFDA treatment caused small insignificant decreases (5-20%) in oxidative burst in response to PMA and bacteria, and phagocytic function was normal. These findings do not correlate with in vivo findings, but conditions for in vitro PFDA treatment were established using spleen cells in the LBT assay, and therefore, conditions may need to be altered in order to mimic in vivo PFDA treatment.

Differences between observed in vivo versus in vitro effects upon PFDA treatment may be explained by differences in exposure time. It is possible that prolonged exposure to PFDA at subtoxic doses in vitro may produce more substantial immunomodulatory effects. There are limits, however, to the length of time primary splenocyte or macrophage cultures can be maintained without transformation or loss of viability.

Based on these data, in vitro experiments cannot totally replace in vivo experiments, but they can provide valuable supplementary information on the immunotoxicity of compounds.

E. GENERAL DISCUSSION

The effect of PFDA on humoral, cellular, and innate immunity in Fischer 344 rats was studied at 8 days and 30 days following a single intraperitoneal injection at doses of 20 mg/kg and 50 mg/kg b.w.. Effects of in vitro PFDA treatment were also determined. Although data were not always statistically significant, certain trends were observed. PFDA was found to significantly decrease IgG_{2a} production at 8 days following PFDA treatment and to cause a trend of decreased DTH responsiveness, reflecting inhibitory effects on humoral and cellular mediated immunity. Innate

immunity was assessed by natural killer cell function and macrophage function. A significant increase in NK activity observed at 50 mg/kg PFDA at 30 but not at 8 days following PFDA treatment. PFDA also suppressed the oxidative burst of macrophages (significant at 20 mg/kg, 8 days following PFDA treatment) and phagocytosis (not statistically significant).

This study examined various mechanisms which could be responsible for the observed immunosuppression using both *in vivo* and *in vitro* exposure methods. A complicating factor in the *in vivo* studies has been the effect of malnutrition on immune responsiveness. PFDA causes anorexia (Andersen *et al.*, 1981), and nutrient deprivation due to decreases in zinc, copper, iodine, selenium, vitamin A and vitamin E have been shown to decrease thymus weight, lymphoproliferation, and DTH responsiveness (Chandra, 1990). Although we controlled for reduced dietary intake by using pair-fed rats, we could not control for metabolic defects in PFDA rats which could exacerbate nutritional deficiencies. One example is the reduction in serum vitamin A levels in PFDA-treated rats which occurs due to decreased hepatic retinyl palmitate hydrolase activity (required for mobilization of vitamin A from the liver (Powers *et al.*, 1986). Alterations in NK activity were probably due to drug-induced anorexia since it occurred in pair-fed animals as well. Others have reported effects of dietary restriction in combination with NK cell activators increasing NK cell activity (Weindruch *et al.*, 1983).

PFDA was found to be cytotoxic as evidenced by decreased relative thymus weight, decreased spleen weight, and decreased lymphocyte numbers. These findings correlate with other reports by Levitt and Liss, (1986) and Liss *et al.*, (1987). The mechanism of cytotoxicity is not directly apparent but appears to be selective for double positive thymocytes, peripheral blood monocytes, peritoneal macrophages, and for splenic T cells in of all phenotypes (CD4+, CD8+ and double positive). B cells and NK cells appear to be relatively more resistant to this toxicity. These findings would suggest interaction of PFDA with specific receptors on certain cell types, whereas others have suggested that PFDA exerts cytotoxicity simply by solubilizing the cell membrane (Levitt and Liss, 1986).

The cytotoxicity due to PFDA treatment is probably partially responsible for the reduction observed in KLH-specific IgG_{2a} production and DTH responsiveness. However, the cytotoxicity cannot be responsible for all of the observed immunosuppression because the remaining viable cells demonstrate differences in function and receptor expression. Although the data were not always statistically significant, certain trends were observed with PFDA treatment. Lymphoproliferation in response to various mitogens was decreased when compared to pair-fed controls. In addition, IL-1 production was frequently found to be altered in PFDA-treated animals, and prostaglandin E production was frequently found to be elevated in PFDA-treated animals, possibly due to PFDA-induced increases in fatty acid oxidation such as has been observed in the liver, and which in turn increases prostaglandin production (Fox, 1981). The combination of these effects would be immunosuppressive (Rappaport and Dodge, 1982) and could result in the decreased immune function produced by PFDA. In addition, MHC Class II antigen expression

was decreased at 8 days following PFDA treatment which would result in decreased presentation of the KLH immunogen, reflected by the reduction in response to KLH. Thirdly, elevations in IL-2 production but decreases in IL-2 receptor expression were observed. In combination with decreased lymphoproliferation, this could indicate defective signal transduction across the IL-2 receptor. It is possible that the observed increase in IL-2 production observed at 50 mg/kg 30 days following PFDA treatment and/or an increase in the percentage of NK cells in the spleen were responsible for enhanced NK activity.

PFDA induced modulation of macrophage function probably occurs following uptake by pinocytosis or phagocytosis as has been demonstrated for other perfluorochemical emulsions (Geyer, 1983). PFDA has been shown to have deleterious effects on electron transport and on mitochondrial oxidases in the liver, severely disrupting normal energy production (Langley, 1990). PFDA may produce similar effects in macrophages, disrupting energy production for phagocytosis and disrupting the NADPH oxidase catalyzed reduction of molecular oxygen which normally results in superoxide anion and ultimately hydrogen peroxide formation. This would explain the decreases in phagocytosis and oxidative burst observed in PFDA-treated animals. In addition, PFDA has been shown to have some direct effects on cell membranes in various cell types (Harrison *et al.*, 1988; Wigler and Shah, 1986; Levitt and Liss, 1987). If macrophages are effected similarly, this could also disrupt phagocytosis.

In vitro PFDA treatment at subtoxic concentrations resulted in decreased IL-2 production and decreased mitogen-induced lymphoproliferation, but there were no significant alterations in receptor expression, IL-1 production, or PGE production. Because IL-2R expression was normal or slightly elevated and IL-2 production was decreased, this may indicate a defect in signal transduction through the IL-2 receptor which may result in decreased lymphoproliferation.

In conclusion, although the data were not always significantly different, certain trends were well supported. PFDA suppressed IgG_{2a} production (8 Days) and DTH responsiveness possibly through combined underlying mechanisms of cytotoxicity, decreased IL-1 and IL-2 production, increased PGE production, and decreased MHC II expression. PFDA also inhibited phagocytosis and the respiratory burst, possibly through mechanisms of membrane alterations, disrupted energy production, and/or disrupted NADPH oxidase activity. Increased NK activity (50 mg/kg, 30 days) appeared to be due to drug-induced anorexia since similar effects were observed in pair-fed animals.

Similar effects have been observed following treatment with the hepatotoxin and immunotoxin TCDD which exhibits similar toxic symptoms to that of PFDA. TCDD impairs antibody responses to both T cell dependent and T cell independent antigens as well as polyclonal B cell activators. TCDD also decreases both delayed type hypersensitivity and cytotoxic T cell generation (Clark *et al.*, 1981).

Future experiments should include determining if signal transduction is defective through the IL-2 receptor. In addition, other tests of cellular immunity can be utilized to support the DTH data. In vivo pathogenicity tests would also be valuable to

determine if the defects in immune response demonstrated here would be sufficient to enhance susceptibility to pathogens. It would also be of interest to measure capacity to produce IFN γ since this cytokine is important in class switching, MHC II upregulation, and macrophage activation. Biochemical studies on macrophages similar to those reported for hepatocytes would test the hypotheses that reduced energy production, electron transport, enzymatic activity and/or direct membrane alteration may be responsible for changes in oxidative burst and phagocytosis exhibited by macrophages.

F. PEROXISOMAL PROLIFERATION IN PFDA TREATED HEPATOCYTES IN VITRO

1. Introduction:

The mechanism by which PFDA initiates peroxisomal proliferation is largely unknown. In other systems, peroxisomal proliferation has been associated with hepatocellular carcinogenesis. Therefore, PFDA induced peroxisomal proliferation may be a useful model to better understand the process and cause of peroxisome formation. Peroxisomes contain peroxidases which can be monitored in live cells using intracellular fluorescent markers. Dihydrorhodamine 123 (DHR-123), when cleaved by peroxidase will fluoresce when interrogated by laser light of 505 wave length. This dye emits 534 wave length light proportional to the peroxidase concentration. Thus fluorescent cytometers such as the Anchored Cell Analysis and Sorting Cytometer (ACAS) may have the potential to measure the formation of peroxisomes in living cells, and to monitor the level of activity.

The purpose of the study was to determine if DHR-123 or other such indicator dyes could be used to measure peroxisome formation in PFDA treated cells.

2. Methods:

a. Cells

Fresh hepatocytes were collected from collagenase perfused rats and provided by the Toxicology Laboratory at Wright Paterson Air Force Base. The hepatocyte cell line, WB-F344, was maintained in MEM medium containing 0.05% MEM 100X vitamin solution, 0.1 % 100X sodium pyruvate, 0.1 % 100X MEM nonessential amino acids, 0.1 % 50X MEM essential amino acids w/o glutamine, 1% pen-strep, 5% heat-inactivated fetal bovine serum and 0.18% 1M HEPES buffer. WB-F344 cells were subcultured every 3-4 days.

b. PFDA Treatment and Cell staining

PFDA-treated or untreated target cells were stained with DHR-123 as follows: Target cells were seeded into Lab-Tek cover glass chambers (Nunc) at a concentration close to confluency for fresh hepatocytes or at a concentration that reached confluency after 3-4 days in culture for WB-F344 cells. PFDA was added at a time prior to cell confluency (day 3 or 4 in culture for WB-F344 cells). DHR-123 solution was added to the cultures on various times after PFDA treatment, and incubated at 37C for 30 min. The chambers were then washed 3X with Delbucco's phosphate buffered saline before image analysis. Between 30 to 160 cells were analyzed per study depending on cell density.

3. Results:

a. Studies with fresh hepatocytes

In preliminary studies fresh hepatocytes from collagenase perfused rat livers were found to be difficult to work with. These tended to detach from the culture vessel and gave inconsistent results. Further studies were performed with WB-F344 cells.

b. Evaluation of a rat hepatocyte cell line

WB-F344 was evaluated for peroxisome analysis. WB-F344 cells were found to attach, grow well in Lab-Tek cover glass chambers (Nunc) and stain nicely with DHR-123.

c. Titration of DHR-123

Initially WB-F344 cells were tested with a series of dilutions of DHR-123. A concentration of 7.5 mM (final concentration) was found to give the best fluorescence with least toxicity.

d. Temporal evaluation fluorescence in PFDA treated hepatocytes

WB-F344 cells seeded in Lab-Tek chambers were treated with 100, 10, 1, 0.1, 0.01 or 0 ug/ml (final concentrations) PFDA in 5% propylene glycol. An untreated control was also included. Cell preparations were stained with DHR-123 on days 1, 2, 3, 4 post treatment and examined by ACAS for fluorescence. Table 1 summarized the results. No direct correlation was found between average fluorescence/cell and PFDA treatment. However, direct examination of images from the treated and untreated cells tended to show occasional brighter fluorescence in cells treated with PFDA than in the untreated controls. Figures 10-15 are examples of the PFDA treated and untreated cells over the 4 day observation period.

TABLE 36 Fluorescence of PFDA Treated and Untreated WB-F344 Cells

Treatment	Day 1	Day 2	Day 3	Day 4
PFDA 0.01 ug/ml	337 (a)	371	382	281
PFDA 0.1 ug/ml	344	327	435	401
PFDA 1 ug/ml	492	241	383	376
PFDA 10 ug/ml	637	380	358	261
PFDA 100 ug/ml	324	257	324	201
PG control	405		589	295
Untreated	450	698	563	318

a. average fluorescence/cell $\times 10^3$

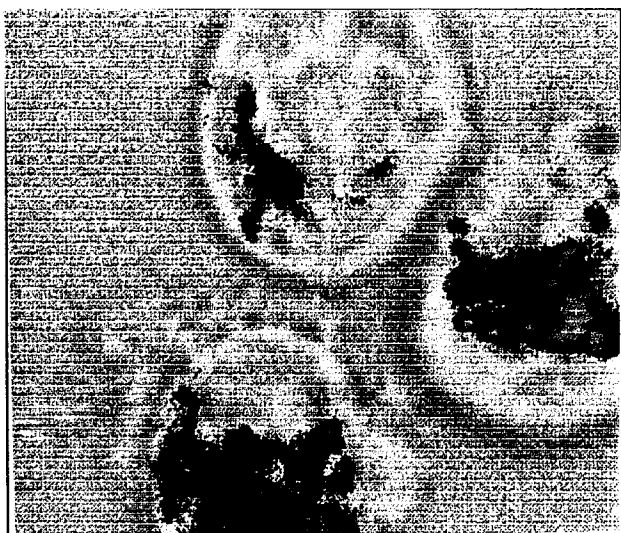


Figure 10 PFDA 0, Day 1

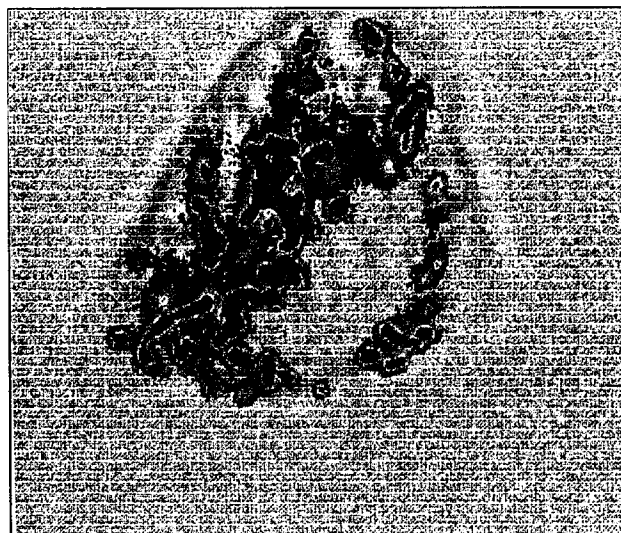


Figure 11 PFDA 100, Day 1



Figure 12 PFDA 0, Day 3

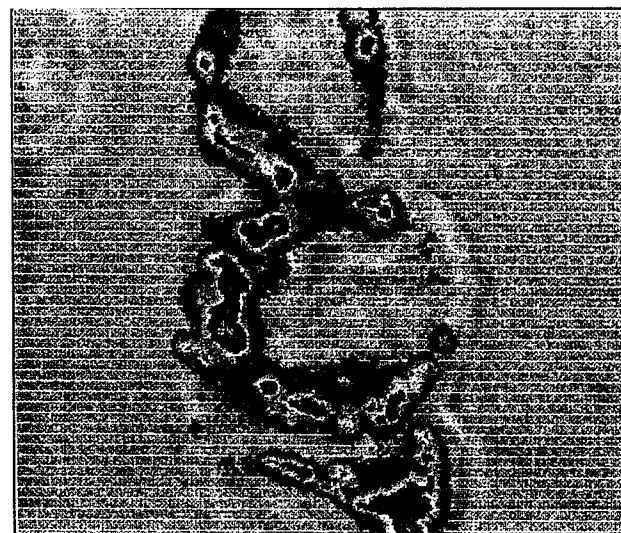


Figure 13 PFDA 100, Day 3



Figure 14 PFDA 0, Day 4

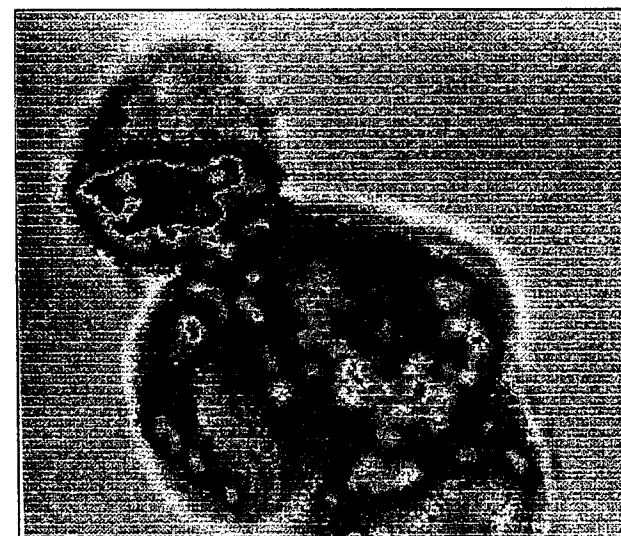


Figure 15 PFDA 100, Day 4

4. Discussion:

After completing the initial study there was concern that the DHR-123 was not measuring peroxisomal peroxidase exclusively. This concern was based on the observation that fluorescence seemed to concentrate in mitochondria of the treated hepatocytes rather than in the newly formed peroxisomes. Additional studies to determine the specificity of the staining procedure were indicated.

G. Production of polyclonal antibodies to enoyl CoA hydratase in chickens.

1. Introduction

One approach to measure peroxisome formation would be to monitor an antigenic marker in target cells where antigen expression is proportional to peroxisome proliferation. The enzyme, enoyl CoA hydratase (ECH) is thought to be a marker for peroxisomes. In this study we attempted to prepare an ECH-specific polyclonal antibody in chickens. Chickens were chosen for antibody production because they are more likely to make antibody to the mammalian ECH than mammalian species such as rabbits or guinea pigs. Once prepared, the antiserum could be used for other studies directed at characterizing the biologic activity of ECH.

2. Method

a. ECH preparation

Two-dimensional gel electrophoresis purified preparations of ECH were provided by Dr. Frank Witzman in acrylamide disks.

b. Immunogen preparation and immunization procedure

The ECH acrylamide disks were emulsified in incomplete Freund's adjuvant prior to injection. Because of the limited amount of ECH available, only 2 chickens were immunized. One subsequently died of causes unrelated to the study.

c. Antibody collection and processing

Unfertilized eggs collected from the immunized chickens were the source of antibody. Egg yolks were pooled and treated with polyethylene glycol (PEG) to precipitate immunoglobulin (IgG) as described by Polsen et al (Immun. Invest. 14:323-327, 1985). The precipitate was solubilized in water and dialyzed against PBS overnight to remove residual PEG.

d. Antibody testing

Antibody was screened for reactivity to ECH by immunoblot analysis. For this procedure, cell extracts from PFDA treated WB-F344 cells were separated by polyacrylamide electrophoresis then electroblotted onto nitrocellulose paper. The paper was then blocked with a solution (blocking solution) containing 5% nonfat dried milk, 0.05% tween-20, and 0.1% sodium azide in PBS, and sliced into strips for storage at -70C. Chicken antibody preparations were diluted 1:100 in blocking buffer and incubated over night at room temp. with the immunoblot strips. The strips were then washed 4X with a solution of 0.05% Tween-20 in PBS (PBS-T). Following washing, the strips were stained with the Vectastain ABC system where biotinylated goat anti chicken IgG served as the secondary reagent and avidin-peroxidase was the tertiary reagent. The chromagen was diaminobenzidine.

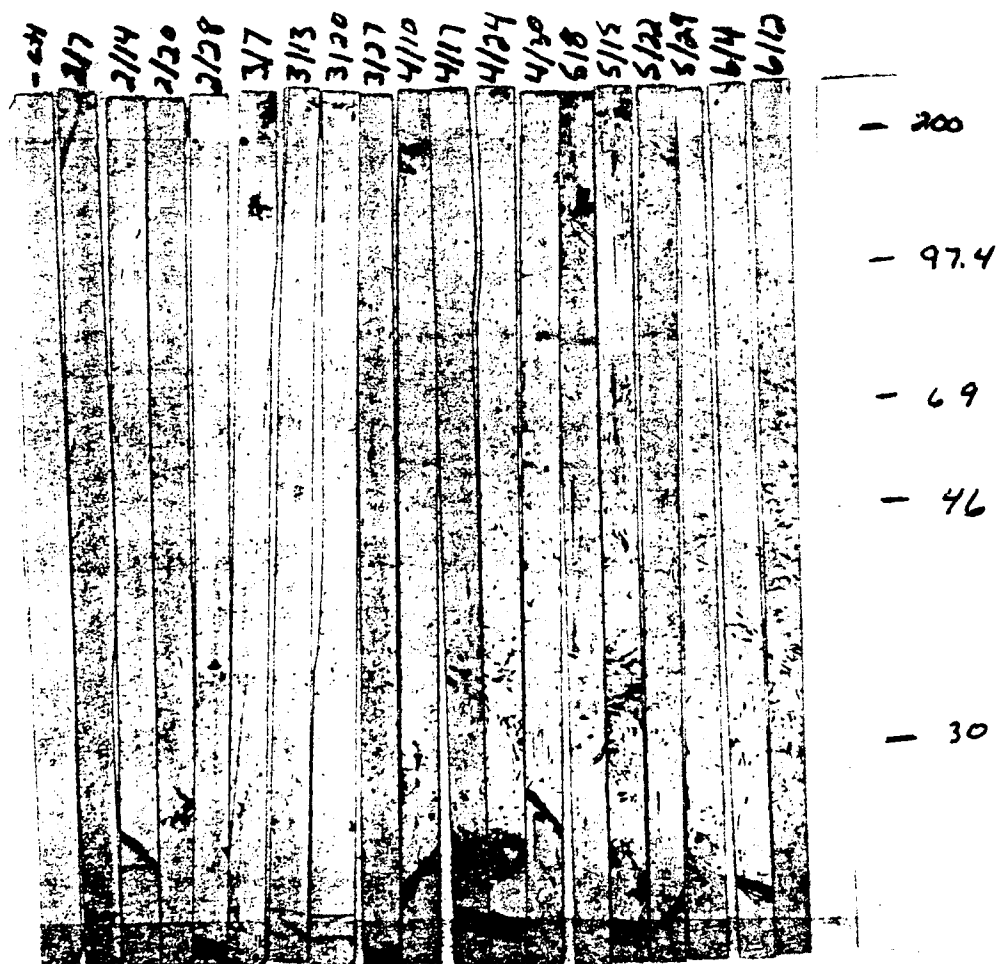
3. Results

Immunoblot analysis showed a faint band corresponding to protein of approximately 80,000 daltons molecular weight. ECH has a molecular weight of 76,000 daltons. To confirm the reactivity with ECH a sample of the IgG extract was sent to Dr. Frank Witzman. Immunoblots using 2 dementional gel blots failed to show any reactivity. Further studies were discontinued because of the ending of the funding period.

4. Discussion

Our results suggest that we were able to produce a relatively weak antibody to a protein with a molecular weight similar to ECH. We were not able to confirm the reactivity was to ECH using 2D electrophoresis immunoblotting but it is possible that the antisera was not of high enough titer to show up in the assay. Further attempt to confirm specificity or to boost the chicken were discontinued.

Figure 16 Immunoblot Of Chicken Anti-ECH



10 DENA L. . . all 1.44te

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